MOLECULAR AND CELLULAR BIOLOGY, Mar. 2004, p. 2103–2117 Vol. 24, No. 5
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.5.2103–2117.2004

Developmentally Essential Protein Flightless I Is a Nuclear Receptor Coactivator with Actin Binding Activity

Young-Ho Lee,[1] Hugh D. Campbell,[2] and Michael R. Stallcup[1,3]*

Departments of Pathology[1] and Biochemistry and Molecular Biology,[3] University of Southern California, Los Angeles, California 90089, and Molecular Genetics and Evolution Group and Centre for the Molecular Genetics of Development, Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia[2]

Received 16 October 2003/Accepted 14 November 2003

Hormone-activated nuclear receptors (NR) activate transcription by recruiting multiple coactivator complexes to the promoters of target genes. One important coactivator complex includes a p160 coactivator (e.g., GRIP1, SRC-1, or ACTR) that binds directly to activated NR, the histone acetyltransferase p300 or CBP, and the arginine-specific histone methyltransferase CARM1. We previously demonstrated that the coactivator function of CARM1 depends both on the methyltransferase activity and on additional unknown proteins that bind to CARM1. In this study a yeast two-hybrid screen for proteins that bind CARM1 identified the protein Flightless I (Fli-I), which has essential roles in *Drosophila* and mouse development. Fli-I bound to CARM1, GRIP1, and NRs and cooperated synergistically with CARM1 and GRIP1 to enhance NR function. Fli-I bound poorly to and did not cooperate with PRMT1, a CARM1-related protein arginine methyltransferase that also functions as an NR coactivator. The synergy between GRIP1, CARM1, and Fli-I required the methyltransferase activity of CARM1. The C-terminal AD1 (binding site for p300/CBP) and AD2 (binding site for CARM1) activation domains of GRIP1 contributed to the synergy but were less stringently required than the N-terminal region of GRIP1, which is the binding site for Fli-I. Endogenous Fli-I was recruited to the estrogen-regulated pS2 gene promoter of MCF-7 cells in response to the hormone, and reduction of endogenous Fli-I levels by small interfering RNA reduced hormone-stimulated gene expression by the endogenous estrogen receptor. A fragment of Fli-I that is related to the actin binding protein gelsolin enhanced estrogen receptor activity, and mutations that reduced actin binding also reduced the coactivator function of this Fli-I fragment. These data suggest that Fli-I may facilitate interaction of the p160 coactivator complex with other coactivators or coactivator complexes containing actin or actin-like proteins.

The nuclear hormone receptors (NRs) are a family of hormone-activated transcriptional activator proteins, which include the receptors for steroid hormones, thyroid hormone, vitamin D, and retinoids (3, 44, 62). NRs bind directly or through other proteins to specific enhancer elements associated with the promoters of target genes and thereby activate or repress transcription. Transcriptional activation involves the recruitment of various coactivator proteins that cause local remodeling of chromatin in the promoter region and help to recruit RNA polymerase II (25, 28, 46).

Many of the coactivators exist or function as complexes of multiple subunits that collaborate to accomplish a single step in transcriptional activation or perform multiple functions that cooperate in a synergistic manner. For example, one relatively well-characterized functional coactivator complex is organized around the p160 coactivators, which include SRC-1, GRIP1/TIF2, and pCIP/ACTR/RAC3/AIB1/TRAM1. The functional and physiological importance of p160 coactivators in NR-mediated transcriptional activation has been established through transient-transfection assays (25, 35, 49), in vitro transcription systems (40), loss of NR function in cells injected with p160 antibodies (60), and specific deficiencies in nuclear receptor-driven developmental processes in mice lacking functional p160 genes (24, 67, 68).

Coactivators generally have two types of functional domains, i.e., those that anchor the coactivator to the promoter and so-called activation domains that transmit the activating signal toward the transcription machinery. Activation domains may propagate the activating signal by catalyzing covalent modifications of histones and other proteins in the transcription machinery or by facilitating transcription initiation through direct protein-protein interactions that anchor other coactivators to the promoter or recruit or activate other components of the transcription machinery. The p160 coactivators are tethered to the promoter by one or more of three NR box motifs with the consensus sequence LXXLL (where L represents leucine and X represents any amino acid) (19, 27, 60, 63). These motifs form amphipathic alpha-helices that insert into a conserved hydrophobic groove located in the AF-2 activation function of the ligand binding domains of all hormone-activated NRs (15, 48). The NR Box motifs are located approximately in the middle of the 1,400-amino-acid polypeptide chain of p160 coactivators. To date two conserved activation domains located near the C terminus of p160 coactivators have been identified as binding sites for additional coactivators: AD1 binds the histone acetyltransferase p300 or CBP (12, 60, 63), while AD2 binds the histone methyltransferase CARM1 (11). The recruitment and coactivator function of CARM1 and p300 (or CBP) apparently depends on the presence of p160

* Corresponding author. Mailing address: Department of Pathology, HMR 301, University of Southern California, 2011 Zonal Ave., Los Angeles, CA 90089-9092. Phone: (323) 442-1289. Fax: (323) 442-3049. E-mail: stallcup@usc.edu.

coactivators (10, 11, 37); therefore, we refer to them as secondary coactivators. In addition to AD1 and AD2, there may be other undefined activation domains of p160 coactivators; for example, the N-terminal region is very highly conserved among the three p160 coactivators (2) but presently has no known role in coactivator function.

CARM1 belongs to the protein arginine methyltransferase family, which is characterized by a highly conserved arginine-specific methyltransferase domain (71). Each of the six members of the mammalian family of proteins methylates a different set of protein substrates (23, 57). For example, CARM1 methylates histone H3 (11), p300/CBP (69), and the RNA binding protein HuR (36), while PRMT1 methylates histone H4 and several RNA binding proteins distinct from HuR (57). After its recruitment to the promoters of steroid hormone-inducible genes, CARM1 methylates histone H3 in the local promoter region as part of the process of transcriptional activation (41). In transient-transfection assays CARM1, but not PRMT1, cooperates synergistically with p300 (or CBP) and p160 coactivators to enhance transcriptional activation by NRs (35). The binding interface between CARM1 and p160 proteins involves an undefined surface on the CARM1 methyltransferase domain and the C-terminal AD2 domain of p160 coactivators (11, 58). Transmission of the activating signal to the transcription machinery by CARM1 involves the methyltransferase activity (11, 35, 41) and additional domains, such as the unique C-terminal and N-terminal domains, which are not involved in the methyltransferase activity (58). This result suggests that CARM1 transmits its activating signal by two cooperative mechanisms, i.e., protein methylation and protein-protein interactions.

We thus propose that additional, presently undefined, proteins are likely to participate in transcriptional activation through protein-protein interactions with GRIP1 and CARM1. We have identified Flightless I (Fli-I) in a yeast two-hybrid screen (22) for proteins which interact with CARM1 and may therefore cooperate with it as coactivators. In *Drosophila*, partial loss-of-function mutations of *fliI* result in a loss of flight ability, while severer mutations lead to impaired cellularization and gastrulation of the embryo (8). Fli-I is a highly conserved protein among diverse vertebrate and invertebrate species (7, 8). The human gene *FLII* is mapped to a region of human chromosome 17p, which is associated with Smith-Magenis syndrome, a genetic disease causing developmental and behavioral abnormalities (13). While it is not yet clear whether Smith-Magenis syndrome is due to haploinsufficiency of *FLII*, homozygous disruption of the orthologous *Fliih* gene in mice leads to very early failure of embryonic development (6). Fli-I is an actin binding protein that can be either associated with the cytoskeleton or in the nucleus, but its exact physiological functions have not been elucidated (16, 17, 26). Since we identified Fli-I as a CARM1 binding protein, we investigated its ability to bind to and collaborate with CARM1 and other NR coactivators in mammalian cells.

MATERIALS AND METHODS

Plasmids. Mammalian cell expression vector pSG5.HA (11), which has simian virus 40 and T7 promoters, was used to express proteins with an N-terminal hemagglutinin (HA) epitope tag. Plasmids encoding the following proteins were previously constructed from pSG5.HA: mouse CARM1, mouse GRIP1, GRIP1N (amino acids 5 to 765), GRIP1 M (amino acids 730 to 1121), GRIP1C (amino acids 1122 to 1462) (11), GRIP1ΔAD1 (lacking amino acids 1057 to 1109), GRIP1ΔAD2 (lacking amino acids 1122 to 1462) (42), GRIP1ΔN (lacking amino acids 1 to 562) (J. H. Kim, H. Li, and M. R. Stallcup, submitted for publication), rat PRMT1 (32), and CARM1(E267Q) with Glu267 changed to Gln (35). The following mammalian cell expression vectors with cytomegalovirus promoters were used: pCMV-p300 encoding full-length p300 (51) and pcDNA3.FliI containing a *Sal*I-*Bam*HI cDNA fragment encoding full-length human Fli-I inserted into the *Xho*I and *Bam*HI sites of pcDNA3. The leucine-rich repeat (LRR) region (amino acids 1 to 494) and gelsolin-like region (amino acids 495 to 1268) of human Fli-I were amplified by PCR with flanking *Mfe*I and *Sal*I sites, digested with these enzymes, and were inserted into the *Eco*RI and *Xho*I sites of pSG5.Flag; pSG5.Flag was derived from pSG5.Flag-TEF4 (5). Mammalian expression vectors for pSG5.flag-Gel.A (human Fli-I amino acids 495 to 827), pSG5.flag-Gel.A(G602S), and pSG5.flag-Gel.A(E585K) were also generated by PCR and site-directed mutagenesis of human Fli-I and were inserted into the *Eco*RI and *Xho*I sites. The expression vector for HA-tagged BAF53 (50) was kindly provided by M. Cole (Princeton University).

Mammalian expression vectors pHE0 encoding human estrogen receptor (ER) α and pCMX.hTRβ1 encoding human thyroid hormone receptor (TR) β1 were described previously, as were the reporter plasmids MMTV(ERE)-LUC for ER, MMTV(TRE)-LUC for TR, and GK1 for Gal4 (11). Mammalian expression vector pM.LRR encoding the Gal4 DNA-binding domain (DBD) fused to the LRR region of Fli-I (amino acids 1 to 494) was constructed by inserting a PCR-amplified cDNA fragment flanked by *Eco*RI and *Sal*I sites into the analogous sites of pM (Clontech).

Bacterial expression vectors encoding glutathione *S*-transferase (GST) fused to GRIP1 fragments were described previously: GRIP1N (amino acids 5 to 765) (30); GRIP1 M (amino acids 730 to 1121) (29); and GRIP1C (amino acids 1122 to 1462) (42). GST fused to ER was encoded by pGEX5X-1.HEG0 (65). Yeast expression vector pGBT9.CARM1 encoding Gal4 DBD fused to full-length CARM1 (amino acids 3 to 608) contained an *Eco*RI insert encoding CARM1.

Protein-protein interaction assays. The following procedures were performed as described previously: yeast two-hybrid screening (11); production of GST fusion proteins in bacteria and binding of labeled proteins translated in vitro to GST fusion proteins bound to glutathione-agarose beads (32); and coimmunoprecipitation and immunoblot experiments (35) with anti-Flag (Sigma F3165), anti-ER and anti-TR (Santa Cruz), and anti-HA (Roche 3F10) antibodies (1 μg of antibody for immunoprecipitation and 1:1,000 dilutions for immunoblots).

Cell culture and transient-transfection assays. Reporter gene activation assays were performed by transient transfection of CV-1 or MCF-7 cells in six-well dishes as described previously (35). Where indicated, 20 nM estradiol (E2) or 2,5,5′-triiodo-L-thyronine (T3) was added to the transfected cultures during the last 40 h of growth.

Peptide antibody production for Fli-I. A peptide representing amino acids 1232 to 1251 in a unique portion of the gelsolin-like region of Flightless-I (human) was synthesized by Tufts University Microchemical Core, and the rabbit antiserum for this peptide (α-Fli-C) was generated by Zymed.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays ware performed according to previously described protocols (41, 43, 56) with minor modifications. MCF-7 cells were cultured for at least 4 days in phenol red-free Dulbecco's modified Eagle medium supplemented with 5% charcoal-dextran-stripped fetal bovine serum. Cells at approximately 90 to 95% confluency were treated with 100 nM E2 or vehicle for 45 min before formaldehyde cross-linking and chromatin preparation. Immunoprecipitation of sonicated chromatin solutions was conducted by overnight incubation at 4°C with the appropriate antibody. After the cross-linking of the immunoprecipitated chromatin was reversed, DNA purification was achieved by phenol-chloroform extraction and ethanol precipitation. The purified DNA was dissolved in 50 μl of 10 mM Tris-HCl, pH 8.0–1 mM EDTA, and 1 μl was used for PCR analysis. For the ChIP and reimmunoprecipitation (Re-IP) assay, the immunoprecipitated fractions were eluted by incubating for 30 min at 37°C in IP elution buffer with 10 mM dithiothreitol (43). The eluates were diluted 50-fold with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.1) and were reprecipitated by specific antibodies. Antibodies used in ChIP assays were obtained from Santa Cruz Biotechnology (normal mouse immunoglobulin G [IgG], normal rabbit IgG; ER, AIB1, p300, and α-Fli-N against the Fli-I LRR region) or Upstate Biotechnology (CARM1). The primers used for detecting portions of the pS2 gene by PCR are listed below from 5′ to 3′ with nucleotide numbers representing the distance from the transcription start site: for the human pS2 promoter region (−31 to −353), GGCAGGCTCTGTTTGCTTAAAGAGCG and GGCCATCTCTCAC TATGAATCACTTCTGC; and for the pS2 coding region (+871 to + 1200),

TGCCAGCTGTGGGGAGCTGAATAACTT and CAGTTCGTTCTGTACA CCGAGGCCACT.

siRNA. Small interfering RNA (siRNA) experiments were performed according to previously published methods (55) with minor modifications. Single-stranded RNAs were synthesized by the University of Southern California Norris Comprehensive Cancer Center Microchemical Core Laboratory. Equimolar concentrations of complementary RNAs were incubated together in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate) at 90°C for 1 min and then at 37°C for 1 h to form a duplex. siRNA duplexes were transfected into MCF-7 cells by using Lipofectamine 2000 or Oligofectamine (Invitrogen) according to the manufacturer's protocol. The siRNA sequences used (listed 5′ to 3′) were as follows: si-FliA, GCUGGAAC ACUUGUCUGUGdTdT and CACAGACAAGUGUUCCAGCdTdT; si-FliB, CAACCUGACCACGCUUCAUdTdT and AUGAAGCGUGGUCAGGUUG dTdT; si-CARM1, GCAGUCCUUCAUCAUCACCdTdT and GGUGAUGAU GAAGGACUGCdTdT; si-AIB1 (55), AGACUCCUUAGGACCGCUUdTdT and AAGCGGUCCUAAGGAGUCUdTdT.

RESULTS

Identification of Fli-I as a CARM1 binding protein by yeast two-hybrid screening. Bait plasmid pGBT9-CARM1, encoding full-length CARM1 fused to Gal4 DBD, and a 17-day-old mouse embryo cDNA library in Gal4 activation domain fusion vector pGAD10 were sequentially transformed into yeast strain Hf7c, which contains the yeast *his3* gene and a *lacZ* gene under the control of Gal4 response elements. Selection of 1.1×10^6 transformed yeast cells on agar plates lacking histidine resulted in the growth of 126 colonies, of which 25 clones were subsequently confirmed as positive by testing for the expression of β-galactosidase. Two of the 25 clones encoded CARM1, which is consistent with previous structural and functional studies indicating that the protein arginine methyltransferases exist as homodimers (45, 71) and which indicates the validity of the bait and the yeast two-hybrid screening conditions for identification of protein partners of CARM1.

Among the other positive cDNA clones, two encoded an N-terminal fragment of the 1,268-amino acid mouse Fli-I protein (extending to codon 480). We used a full-length human cDNA clone (8) for all studies described below. The N-terminal region of Fli-I contains an LRR domain consisting of 16 tandem copies of a 23-amino-acid leucine-rich motif (Fig. 1A) (8, 31). LRR domains have been implicated in protein-protein interactions. The central and C-terminal regions of the Fli-I polypeptide chain are homologous to the gelsolin family of actin binding proteins (34). Fli-I binds actin and colocalizes with the actin cytoskeleton under certain conditions but can also localize to the nucleus under other conditions (16, 17). These studies suggest that Fli-I may have a nuclear function and in addition may be involved in signal-regulated remodeling of or coupling of other proteins to actin or the actin cytoskeleton. Since we identified Fli-I in our two-hybrid screen by its ability to bind the NR coactivator CARM1, we confirmed the binding by other methods and investigated the ability of Fli-I to cooperate with CARM1 and other NR coactivators in enhancing transcriptional activation by NRs.

Specific binding of Fli-I to CARM1 and NRs in vitro and in vivo. Fli-I protein translated in vitro was tested for its ability to bind GST-CARM1 immobilized on glutathione-agarose beads (Fig. 1B). As reported previously (17), production of full-length (145-kDa) Fli-I in this system was relatively inefficient (lane 1). There was relatively weak but specific binding of full-length Fli-I to GST-CARM1 (lane 3), compared with GST alone (lane 2), and full-length Fli-I was enriched over the smaller bands when the input and CARM1-bound fractions were compared. These data suggest that Fli-I binds directly to CARM1.

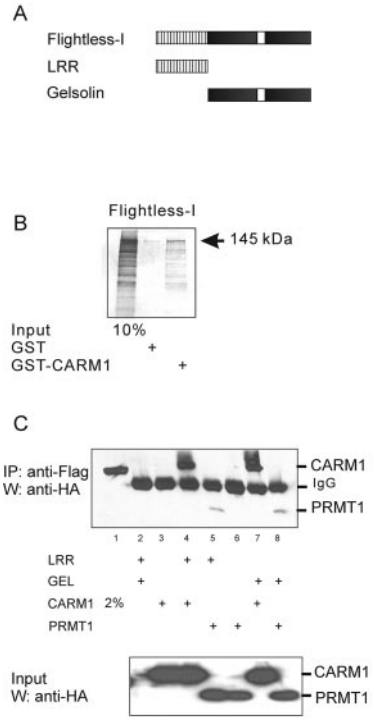

FIG. 1. Binding of CARM1 to Fli-I in vitro and in vivo. (A) The LRR region (vertical stripes) and two gelsolin-like motifs (black boxes) of Fli-I protein are indicated. (B) In vitro binding assay. [^{35}S]Fli-I protein synthesized in vitro was incubated with GST or GST-CARM1 immobilized on glutathione-agarose beads; bound proteins were eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autofluorography. A portion of the in vitro translated Fli-I protein before incubation with the beads is shown at left (Input, 10%). The position of the 145-kDa full-length Fli-I protein is indicated. (C) Coimmunoprecipitation assay. pSG5.Flag-FliI(LRR) or pSG5.Flag-FliI(gelsolin) (2.5 μg) was transiently transfected along with pSG5.HA-CARM1 or pSG5.HA-PRMT1 (2.5 μg) into Cos-7 cells. Immunoprecipitation (IP) was performed on transfected-cell extracts with antibodies against the Flag epitope, and the precipitated proteins were analyzed by immunoblotting (W) with anti-HA antibodies (upper panel). A sample of the transfected-cell extract before immunoprecipitation (2% of the volume used for the immunoprecipitation shown in lane 3) is shown in lane 1. The relative expression of HA-CARM1 and HA-PRMT1 before immunoprecipitation is shown in the lower panel. Results are representative of two independent experiments. LRR, LRR fragment of Fli-I; GEL, gelsolin-like fragment of Fli-I; and IgG, position of the immunoglobulin heavy chain on the immunoblot.

To achieve more efficient expression and to test the properties of specific Fli-I subdomains, Fli-I was subsequently divided into two separate fragments: amino acids 1 to 494, containing the LRR region; and amino acids 495 to 1268, containing the gelsolin-like domains. To test interactions in vivo, the Fli-I fragments fused to a Flag epitope were expressed by transient transfection in Cos-7 cells along with HA epitope-tagged CARM1 and coimmunoprecipitation experiments were performed on the transfected-cell extracts by using antibodies against the Flag epitope and subsequently analyzing the im-

munoprecipitates by immunoblot with antibodies against the HA epitope (Fig. 1C, upper panel). When CARM1 was expressed without any Fli-I fragment, it was not detected in the immunoprecipitates (lane 3); however, CARM1 coimmunoprecipitated with the N-terminal LRR domain of Fli-I (lane 4) as well as the C-terminal gelsolin-like domain of Fli-I (lane 7), suggesting the presence of at least two direct or indirect CARM1 binding sites on the Fli-I protein.

To test the specificity of the interaction between Fli-I and CARM1, another member of the protein arginine methyltransferase family, PRMT1 (38), was substituted for CARM1. There is substantial homology between CARM1 and PRMT1 in their 320-amino-acid methyltransferase domains (71), and both proteins can function as NR coactivators in cooperation with p160 coactivators (32). PRMT1 specifically coprecipitated with both fragments of Fli-I (Fig. 1C, upper panel, lanes 5, 6, and 8) but much more weakly than CARM1 (lanes 4 and 7). Immunoblots performed with anti-HA antibodies on samples of the transfected-cell extracts taken before immunoprecipitation showed that CARM1 and PRMT1 were expressed at similar levels (Fig. 1C, lower panel).

Since Fli-I binds to NR coactivator CARM1, we also tested whether Fli-I may bind to NRs. Unlike full-length Fli-I (Fig. 1B), ^{35}S-labeled LRR and gelsolin-like domains of Fli-I were synthesized efficiently in vitro (Fig. 2A, lanes 1 and 5). The LRR and gelsolin-like fragments did not bind to GST (lanes 2 and 6) and bound very weakly to a GST-ER fusion protein in the absence of E2 (lanes 3 and 7). The interaction of both Fli-I fragments with GST-ER was strongly enhanced by E2 (lanes 4 and 8). Coimmunoprecipitation experiments were used to test for the same interactions in Cos-7 cells transiently expressing ER with each Flag-tagged Fli-I fragment. When the Fli-I fragments or ER was expressed alone, anti-Flag antibodies did not precipitate ER (Fig. 2B, upper panel, lanes 1 and 2). However, when ER was expressed with the LRR fragment or the gelsolin-like fragment of Fli-I, ER was coprecipitated by anti-Flag antibody (lanes 3 to 6). Moreover, in contrast to the binding in vitro (Fig. 2A), the association between ER and the Fli-I fragments in vivo occurred in the presence or absence of E2 (Fig. 2B, upper panel, lanes 3 to 6). Since the binding of Fli-I fragments to ER was hormone dependent in vitro but not in vivo, additional cellular factors may stabilize the interaction between ER and Fli-I in vivo, especially in the absence of hormone. Alternatively, the overexpression of the proteins in transient transfections could cause the hormone-independent association.

Interaction of the Fli-I fragments with another NR, TR, was also tested by coimmunoprecipitation; in this case, antibodies against TR were used for immunoprecipitation, and antibodies against the Flag epitope tag were used to visualize the coprecipitated Fli-I fragments. As with ER, TR coprecipitated with both the LRR fragment and the gelsolin-like fragment of Fli-I in a hormone-independent manner (Fig. 2C, upper panel, lanes 1 to 6). The association of the Fli-I fragments with CARM1 was used as a positive control (lanes 7 and 8).

Recruitment of endogenous Fli-I protein to a steroid hormone-regulated promoter in response to hormone. The association of Fli-I with NRs and several NR coactivators suggests that Fli-I may participate in the NR-dependent transcriptional regulation process. Therefore, ChIP assays were performed to test whether endogenous Fli-I protein is recruited to an NR-regulated promoter in response to the appropriate hormone. For this purpose, two antibodies against Fli-I, one commercially available mouse monoclonal against the LRR region and one custom-made rabbit peptide-directed antiserum against a unique portion of the gelsolin-like region, were shown by immunoblot and immunoprecipitation assays to recognize specifically the Fli-I protein (data not shown; also see Fig. 7B). Endogenous Fli-I protein was detected in MCF-7 breast cancer

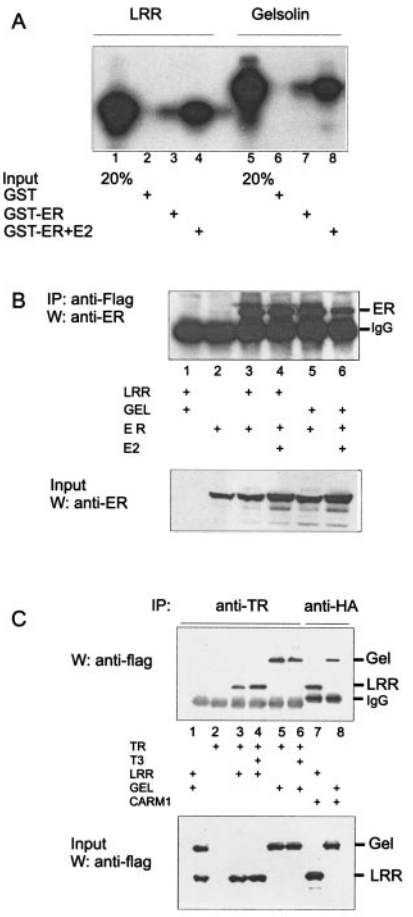

FIG. 2. Binding of NRs to Fli-I in vitro and in vivo. (A) In vitro binding with ER. LRR and gelsolin-like fragments of Fli-I were translated in vitro and were tested for binding to bead-bound GST or GST-ER in the absence or presence of E2. (B) Coimmunoprecipitation with ER. Flag-tagged LRR or gelsolin-like fragments of Fli-I (2.5 μg of plasmid) were coexpressed with ER (2.5 μg of plasmid) in Cos-7 cells in the presence or absence of E2. Immunoprecipitation (IP) by anti-Flag antibody was followed by immunoblotting (W) with anti-ER antibody (upper panel). Expression of ER in the transfected-cell extracts before immunoprecipitation (input) was examined by immunoblotting with anti-ER antibodies (lower panel). (C) Coimmunoprecipitation with TR and CARM1. Coimmunoprecipitation of the Fli-I fragments with TR (2.5 μg of plasmid) or HA-tagged CARM1 (2.5 μg of plasmid) was tested as done for panel B in the presence and absence of T3, except that anti-TR (lanes 1 to 6) or anti-HA (lanes 7 and 8) was used for immunoprecipitation, and Fli-I fragments were detected on the subsequent immunoblot (W) with anti-Flag antibodies. Expression of LRR and gelsolin-like fragments of Fli-I in the transfected-cell extracts was examined before immunoprecipitation (input) by immunoblot with anti-Flag antibodies (lower panel).

cells (data not shown), which contain the estrogen-inducible pS2 gene. When MCF-7 cells were treated with 100 nM E2 or were left untreated for 45 min and were analyzed by ChIP analysis, ER protein was recruited to pS2 promoter in a hormone-dependent manner (Fig. 3A and B). Similarly, hormone-dependent recruitment of Fli-I protein to the pS2 promoter region was observed when 0.5 μg of the mouse monoclonal antibody (Fig. 3A) or 5 μl of the rabbit antiserum (Fig. 3B) against Fli-I was used; however, when twice as much of either anti-Fli-I antibody was used, Fli-I protein appeared to be constitutively associated with the pS2 promoter (Fig. 3A, last two lanes, and B, not shown). These results suggest that small amounts of Fli-I protein are associated with the pS2 promoter even in the absence of hormone but that promoter occupancy by Fli-I is increased by E2 treatment. Normal IgG served as a negative control for the immunoprecipitations to demonstrate the specificity of the Fli-I antibodies (Fig. 3A and B). When PCR primers recognizing a portion of the coding sequence (nucleotides 871 to 1200, relative to the transcription start site) of the pS2 gene were used to test the same chromatin fractions immunoprecipitated with the antibodies against ER or Fli-I, no amplification products were observed (Fig. 3B, bottom panel), indicating that the recruitment of both ER and Fli-I is specific for the pS2 promoter, not for the coding region of the pS2 gene. Thus, both ER and Fli-I appear in the transcription initiation complex but do not appear to travel along with RNA polymerase as it progresses through the coding region. The recruitment of Fli-I protein to the pS2 promoter could be through the binding of Fli-I to ER or to one of the other coactivators, such as GRIP1 or CARM1.

While these ChIP data indicate that both ER and Fli-I are recruited to pS2 promoters in the cell culture in response to hormone, they do not indicate whether Fli-I and ER molecules are present simultaneously on any individual pS2 promoter. ChIP and Re-IP assays were used to answer this question: first, the ChIP protocol was performed with the anti-ER antibody; the precipitated ER-containing chromatin fractions were eluted from the antibody-bead complex with dithiothreitol, diluted, and reimmunoprecipitated by using the two different antibodies against Fli-I or antibodies against a known ER-associated coactivator. DNA was recovered from the second immunoprecipitated pellet and was analyzed by PCR with primers against the pS2 promoter as before (Fig. 3C). By this analysis, ER was shown to be simultaneously localized on individual hormone-activated pS2 promoters with Fli-I as well as with each of three known ER coactivators, AIB1, CARM1, and p300. When the ChIP and Re-IP procedure was performed with the anti-Fli-I antibody for the first immunoprecipitation, Fli-I was shown to coincide with ER, AIB1, and CARM1 on individual hormone-activated pS2 promoters, and CARM1 co-localized with Fli-I even on hormone-independent promoters (Fig. 3D). Since high levels of the Fli-I antibodies were used in this experiment (Fig. 3D), the hormone-independent binding of Fli-I and CARM1 to the pS2 promoter observed in this experiment is consistent with the results observed with high levels of Fli-I antibodies in standard ChIP assays (Fig. 3A). The ChIP and Re-IP results indicate that endogenous Fli-I is part of a large ER-associated coactivator complex assembled on the hormone-activated pS2 promoter.

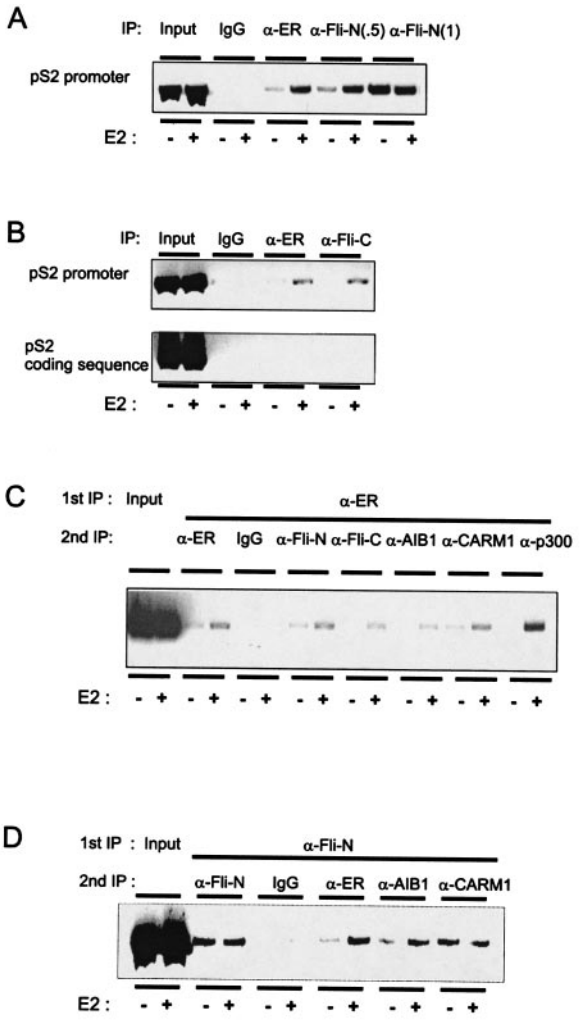

FIG. 3. Estrogen-dependent recruitment of endogenous Fli-I to the endogenous pS2 promoter in MCF-7 cells. (A and B) ChIP assay. MCF-7 cells were incubated with or without E2 hormone for 45 min. Extracted chromatin was incubated with antibodies against the indicated proteins, and DNA purified from the immunoprecipitated (IP) fractions was analyzed by PCR with primers for the pS2 promoter or coding region. Mouse monoclonal antibody against the Fli-I LRR region (α-Fli-N) was used at 0.5 or 1 μg per assay, rabbit antiserum against a peptide from the gelsolin-like region of Fli-I (α-Fli-C) was used at 5 μl per assay, and all other antibodies were used at 1 μg per assay, including normal mouse or rabbit IgG. The results presented here are representative of seven independent experiments. (C) Simultaneous binding of pS2 promoter by ER and several coactivators. Chromatin from hormone-treated or untreated MCF-7 cells was immunoprecipitated first with anti-ER antibody; immunoprecipitates were eluted from the beads and were immunoprecipitated again with antibodies against the specified proteins. Amounts of antibodies used: 0.5 μg of α-Fli-N, 5 μl of α-Fli-C, and 1 μg of all other antibodies. DNA purified from the immunoprecipitated fractions was analyzed by PCR with primers for the pS2 promoter. (D) Simultaneous binding of pS2 promoter by Fli-I and ER or other coactivators. MCF-7 cell chromatin (E2 treated or untreated) was immunoprecipitated first by 1 μg of α-Fli-N; immunoprecipitates were eluted from the beads and were immunoprecipitated again with 1 μg of antibodies against the specified proteins. DNA purified from the immunoprecipitated fractions was analyzed by PCR with primers for the pS2 promoter.

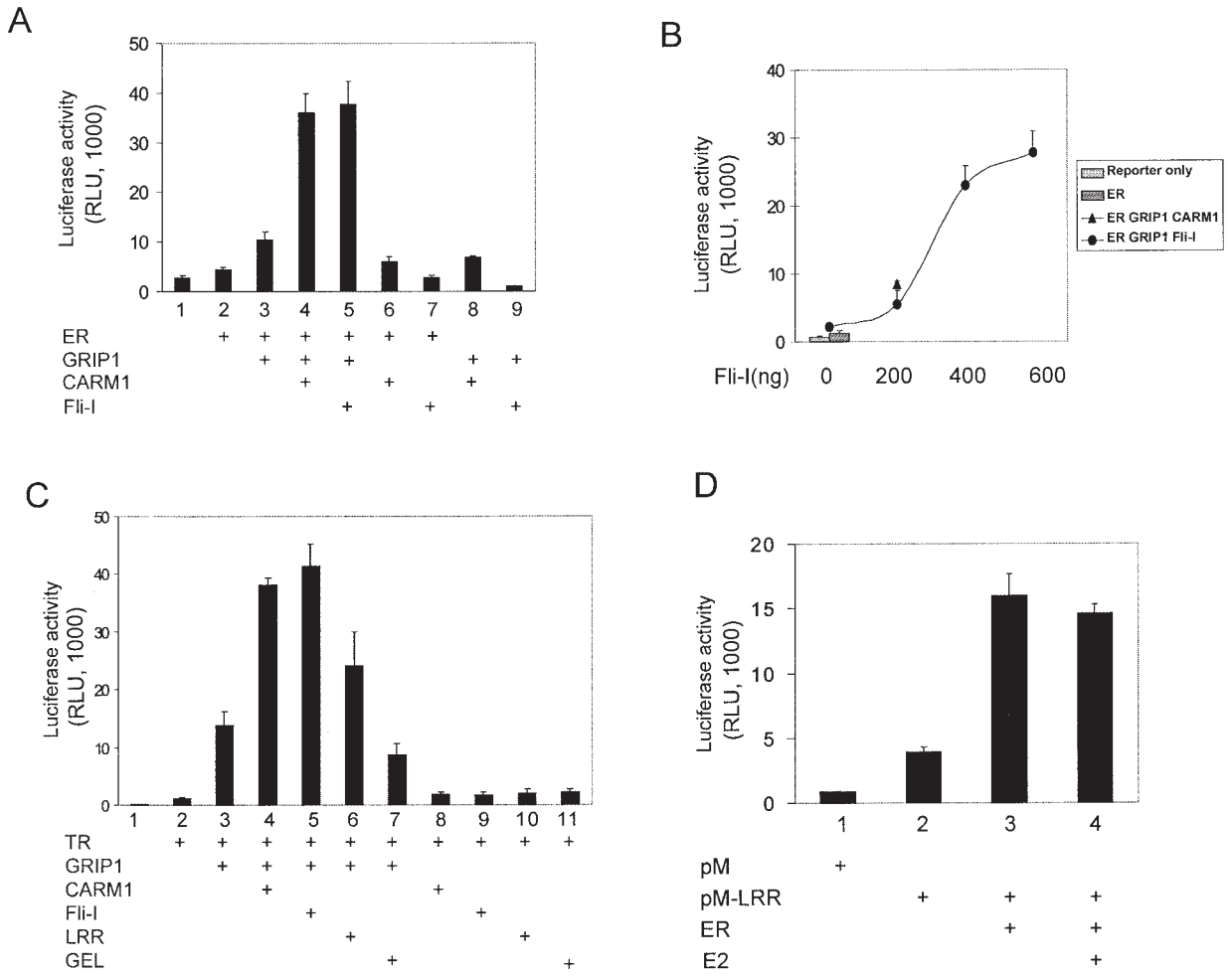

FIG. 4. Fli-I is a secondary coactivator for NRs. (A) Dependence of Fli-I coactivator activity on GRIP1 and ER. CV-1 cells were transfected with MMTV(ERE)-LUC reporter plasmid (250 ng) and with expression vectors encoding ER (100 ng), GRIP1 (250 ng), CARM1 (500 ng), and Fli-I (500 ng) as indicated elsewhere. Transfected cells were grown with E2, and luciferase activities of the transfected-cell extracts were determined. (B) Increasing coactivator activity with increasing Fli-I. Transfections were performed as done for panel A except that 200 ng of CARM1 vector and 200 to 600 ng of Fli-I vector were used. (C) Partial coactivator function by the Fli-I LRR domain. Transfections were performed with MMTV(TRE)-LUC reporter plasmid (250 ng) and expression vectors encoding TR (100 ng), GRIP1 (250 ng), CARM1 (500 ng), and full-length Fli-I or the LRR or gelsolin-like fragment (500 ng). Transfected cells were grown with T3 before harvesting for luciferase assays. Similar results were obtained with ER and the MMTV(ERE)-LUC reporter plasmid (data not shown). (D) Autonomous activation function in the Fli-I LRR domain. CV-1 cells were transfected with GK1 reporter plasmid (250 ng) encoding luciferase controlled by Gal4 response elements and expression vectors encoding Gal4 DBD or Gal4 DBD fused to the LRR domain (250 ng) and ER (500 ng). Transfected cells were grown with E2 as indicated. Results portrayed in panels A to D are representative of three or more independent experiments.

Fli-I is a secondary coactivator for NRs. Since Fli-I binds to NRs and the NR coactivator CARM1 and since Fli-I is recruited to the pS2 promoter in response to hormone activation, we tested the ability of Fli-I to serve as a coactivator for NRs, i.e., to enhance the ability of NRs to activate expression of reporter genes containing NR-responsive enhancer elements. We have previously defined two different sets of transient-transfection conditions for testing the function of NR coactivators (32, 35). When standard, relatively high levels of NR expression vector are transfected into CV-1 cells, the NR alone exhibits hormone-dependent activation of a suitable reporter gene. The effects of some individual coactivators and some pairs of coactivators can also be observed, but addition of a third coactivator fails to elicit any further enhancement of reporter gene activity. In contrast to these high-NR conditions, use of much lower levels of NR expression vectors in the transfection results in much more stringent coactivator requirements. Under low-NR conditions, the NR alone effects little or no reporter gene expression; instead, specific combinations of three coactivators are required along with the NR and its hormone for efficient reporter gene expression.

When CV-1 cells were transfected with high levels of ER expression vector (100 ng per well of a six-well dish), GRIP1 enhanced the ability of ER to activate reporter gene expression in the presence of E2 (Fig. 4A, assays 1 to 3). Addition of CARM1 or Fli-I along with GRIP1 further enhanced reporter gene expression (assays 4 and 5). The ability of CARM1 and Fli-I to function as coactivators depended on the presence of

both ER and GRIP1 (assays 6 to 9). Thus, as previously shown for CARM1 (10, 11), Fli-I functioned as a secondary coactivator; i.e., its ability to enhance NR function depended on the coexpression of a p160 coactivator such as GRIP1. In the presence of ER and GRIP1, the degree of enhancement of reporter gene activity by Fli-I was roughly proportional to the amount of Fli-I expression vector transfected (Fig. 4B). Under similar high-NR conditions, Fli-I also functioned as a secondary coactivator for TR (Fig. 4C, assays 1 to 5 and 8 and 9). Since the separate LRR and gelsolin-like domains of Fli-I each bound to CARM1 and NRs, we also tested the ability of these Fli-I fragments to replace full-length Fli-I as coactivators. The LRR fragment exhibited a weak GRIP1-dependent coactivator activity, compared with full-length Fli-I, but the gelsolin-like fragment had no coactivator activity (Fig. 4C, assays 6 and 7 and 10 and 11). It is worth noting that the two fragments of Fli-I are expressed at much higher levels in transient transfections than full-length Fli-I (data not shown); when this is taken into account, full-length Fli-I is a much more powerful coactivator than the LRR fragment of Fli-I, and thus, the efficient coactivator function of Fli-I depends upon both the LRR and gelsolin-like domains.

Autonomous activation function in the LRR domain of Fli-I. After coactivators are recruited to the promoter, they can transmit an activating signal to the transcription machinery via an enzymatic activity (e.g., various histone modifications) or through protein-protein contacts with downstream components of the transcription machinery, such as other coactivators, chromatin components, basal transcription factors, or RNA polymerase II. In some but not all cases, such signal transduction domains of coactivators have an autonomous activation function that can activate a reporter gene when the activation function is fused to Gal4 DBD and the fusion protein is expressed with a reporter gene controlled by Gal4 response elements (10, 32, 58). When the LRR domain of Fli-I was fused to Gal4 DBD, it enhanced expression of a Gal4-responsive reporter gene (Fig. 4D, assays 1 and 2). Coexpression of ER substantially enhanced this activity in a hormone-independent manner (assays 3 and 4), which corresponds to the hormone-independent binding of Fli-I to ER in coimmunoprecipitation studies (Fig. 2B). These results suggest that the autonomous activation function of Fli-I may contribute to the coactivator function of Fli-I through interaction with downstream components of the transcription machinery; they further suggest the functional relevance of the binding of Fli-I to NRs.

Coactivator synergy between CARM1 and Fli-I. Since CARM1 and Fli-I each functions as a secondary NR coactivator in cooperation with GRIP1 (Fig. 4) and since CARM1 and Fli-I specifically bind to each other (Fig. 1), we tested whether CARM1 and Fli-I can cooperate as coactivators for NRs. We employed the transient-transfection assay system with low levels of NR expression vector, since we previously established that these low-NR conditions are characterized by stringent requirements for multiple coactivators and facilitate the observation of synergistic interactions among multiple coactivators (35). In this series of experiments with TR, all transfected cultures were exposed to thyroid hormone, since we previously demonstrated that all coactivator activities depend on the presence of NR and the appropriate hormone. Also as shown previously for these low-NR conditions (35), TR alone did not activate the reporter gene and coexpression of GRIP1 with TR caused little or no activation (Fig. 5A, assays 1 to 3). Coexpression of GRIP1 with one other coactivator (CARM1, p300, Fli-I, or Fli-I fragments) also caused little or no enhancement of TR-mediated reporter gene expression (assays 4 to 8). However, the coexpression of GRIP1 and CARM1 with p300 or Fli-I resulted in a dramatic synergistic enhancement of TR function (assays 9 and 10). Both domains of Fli-I were required for the GRIP1–CARM1–Fli-I coactivator synergy (assays 10 to 12).

While the GRIP1–CARM1–Fli-I combination was synergistic, substitution of p300 or PRMT1 for CARM1 failed to produce synergy (Fig. 5B, assays 8 to 10). The synergy between CARM1 and Fli-I was completely dependent on the coexpression of GRIP1 (assay 11), confirming the roles of CARM1 and Fli-I as secondary coactivators. Thus, Fli-I specifically cooperated with CARM1 as a coactivator and did not cooperate with p300 or PRMT1. The functional cooperation of Fli-I with CARM1 but not PRMT1 correlates with the binding specificity of Fli-I with CARM1 but not PRMT1 (Fig. 1) and thus suggests that the binding of CARM1 and Fli-I may be important for their functional cooperation. Although p300 and PRMT1 failed to cooperate functionally with Fli-I (Fig. 5B), they were still capable of functioning synergistically with other combinations of coactivators: p300 cooperated synergistically with CARM1 and GRIP1 (Fig. 5A, assay 9) (35) and PRMT1 also functioned synergistically with CARM1 and GRIP1 (Fig. 5C, assay 11) (32). Thus, the low-NR transient-transfection system revealed functional collaborations between specific combinations of coactivators: when coexpressed with GRIP1, CARM1 cooperated with p300 (Fig. 5A) (35), with Fli-I (Fig. 5A to C), and with PRMT1 (Fig. 5C) (32), but Fli-I did not cooperate with p300 (Fig. 5B) or PRMT1 (Fig. 5B), and PRMT1 did not cooperate with p300 (35).

To test the role of CARM1's protein arginine methyltransferase activity in the GRIP1–CARM1–Fli-I synergy, a CARM1(E267Q) mutant was substituted for CARM1 in the low-NR transient-transfection system. CARM1(E267Q) lacks protein methyltransferase activity but retains the ability to bind GRIP1, and substitution of this mutant for wild-type CARM1 almost completely eliminated the GRIP1-CARM1-p300 coactivator synergy (35). The E267Q mutation also eliminated the GRIP1–CARM1–Fli-I synergy with ER (Fig. 5C, assays 8 and 9) and with TR (data not shown), indicating that the CARM1 methyltransferase activity is essential for the coactivator synergy between CARM1 and Fli-I. The failure of PRMT1 and the CARM1(E267Q) mutant to cooperate with Fli-I was not due to lack of their expression or to improper folding of the CARM1 mutant, because PRMT1 exhibited synergy with wild-type CARM1 and because the CARM1 mutant retained partial synergistic activity with PRMT1, even though the mutant was inactive in combination with Fli-I (Fig. 5C, assays 9 to 12). Thus, the CARM1 methyltransferase activity was essential for synergistic cooperation of CARM1 with Fli-I but was less important for synergistic cooperation of CARM1 with PRMT1.

Coactivator function of Fli-I depends on the N-terminal domain of GRIP1. Since the coactivator function of Fli-I was completely dependent on coexpression of GRIP1, we tested whether Fli-I binds to GRIP1 and whether such an interaction

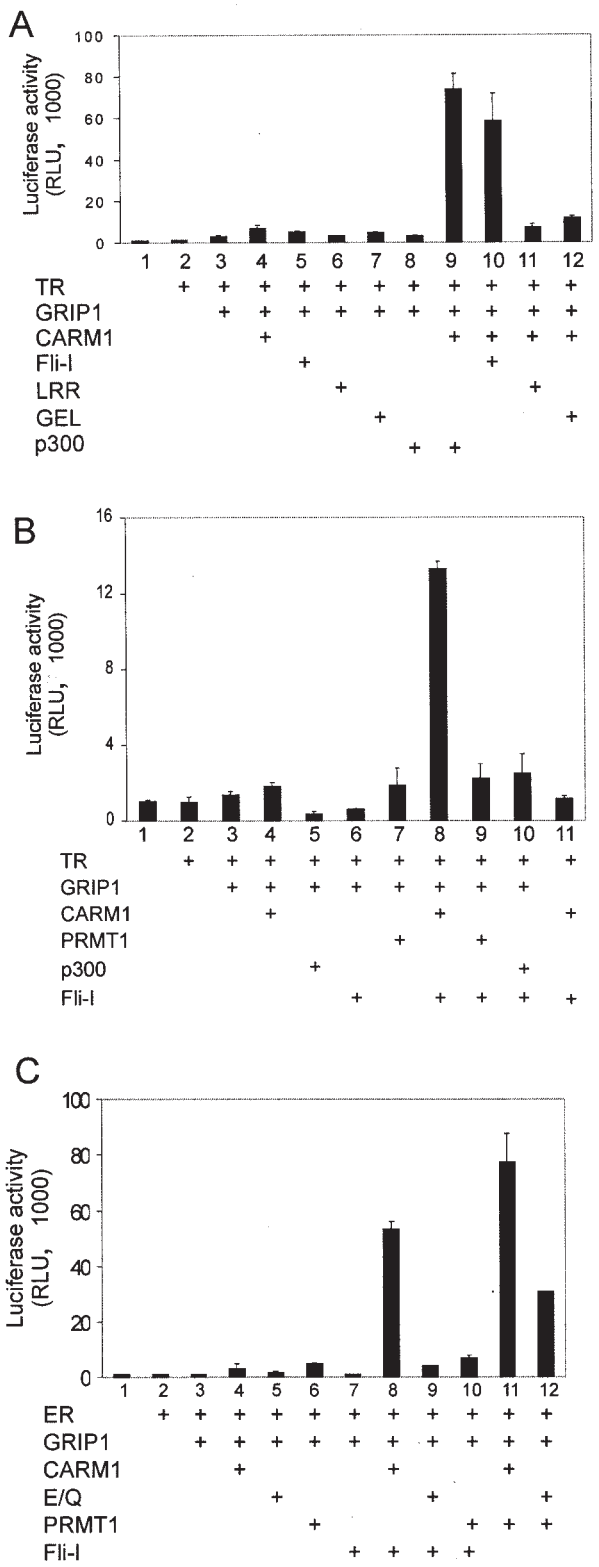

FIG. 5. Synergistic coactivator function between Fli-I and CARM1. CV-1 cells were transfected with MMTV(TRE)-LUC (250 ng) (A and B) or MMTV(ERE)-LUC (250 ng) (C) and expression vectors encoding TR (1 ng) or ER (1 ng), GRIP1 (250 ng), CARM1 wild type or E267Q mutant (500 ng), PRMT1 (500 ng), p300 (500 ng), and full-length Fli-I or the LRR or gelsolin-like fragment (500 ng). (A) Full-length Fli-I required for synergy. (B) Fli-I synergy with CARM1 but not with PRMT1 or p300. (C) Methyltransferase activity of CARM1 required for synergy with Fli-I. Transfected cells were grown with T3 (A and B) or E2 (C) before harvest. Results in each panel are representative of three or more independent experiments.

might explain the functional coactivator relationship between GRIP1 and Fli-I. Three fragments of GRIP1 were prepared as GST fusion proteins: GRIP1.N, amino acids 5 to 730, containing the basic helix-loop-helix (bHLH) and two Per-ARNT-SIM (PAS) domains as well as the first two (of three) LXXLL motifs; GRIP1.M, amino acids 730 to 1121, containing the third LXXLL motif and the AD1 domain, which binds CBP and p300; and GRIP1.C, amino acids 1122 to 1462, containing the AD2 domain, which binds CARM1. The Fli-I LRR fragment translated in vitro bound specifically to immobilized GST-GRIP1.N (Fig. 6A, upper panel, lane 3), but the gelsolin-like fragment bound very weakly to the GRIP1 N-terminal region (lane 8). Only weak background-level binding of both Fli-I fragments was observed with GST (lanes 2 and 7), GST-GRIP1.M (lanes 4 and 9), or GST-GRIP1.C (lanes 5 and 10). As a positive control, we demonstrated that CARM1 translated in vitro bound to GST-GRIP1.C (Fig. 6A, lower right panel), as shown previously (11). These results suggest a direct interaction between the LRR domain of Fli-I and the N-terminal region of GRIP1.

To test for Fli-I-GRIP1 interactions in vivo, HA-tagged GRIP1 fragments were expressed by transient transfection in Cos-7 cells along with Flag-tagged Fli-I fragments. Immunoprecipitations were performed with anti-Flag antibodies, and coprecipitated GRIP1 fragments were visualized by immunoblot with antibodies against the HA epitiope. As in the in vitro binding studies (Fig. 6A), the coimmunoprecipitation experiments demonstrated that the GRIP1 N-terminal region bound to the Fli-I LRR domain but not to the Fli-I gelsolin-like domain (Fig. 6B, lanes 5 and 8). However, while the C-terminal region of GRIP1 did not bind to either Fli-I domain in vitro (Fig. 6A), it bound to both domains of Fli-I in vivo (Fig. 6B, lanes 7 and 10). The middle domain of GRIP1 did not bind to either Fli-I domain (lanes 6 and 9). The combined results from the in vitro and in vivo binding studies suggest that the Fli-I LRR domain binds directly to the N-terminal region of GRIP1, while both the LRR and gelsolin-like domains of Fli-I bind indirectly to the C-terminal region of GRIP1. Since CARM1 binds to the C-terminal region of GRIP1 and since both the LRR and gelsolin-like domains of Fli-I can bind to CARM1, we propose that the binding of Fli-I to the GRIP1 C-terminal region observed in vivo is mediated through endogenous CARM1, which is present at relatively high levels in Cos-7 cells (data not shown).

While Fli-I physically interacts with NRs, CARM1, and GRIP1, the functional dependence of Fli-I on GRIP1 (Fig. 4 and 5) led us to test whether the observed binding of Fli-I to the N-terminal region of GRIP1 (Fig. 6A and B) is important for the functional collaboration between these two coactivators. We also tested whether other known functional domains of GRIP1 are required for GRIP1–CARM1–Fli-I coactivator synergy. Deletion mutants of GRIP1 lacking the N-terminal domain (ΔN, lacking amino acids 1 to 562), the AD1 domain

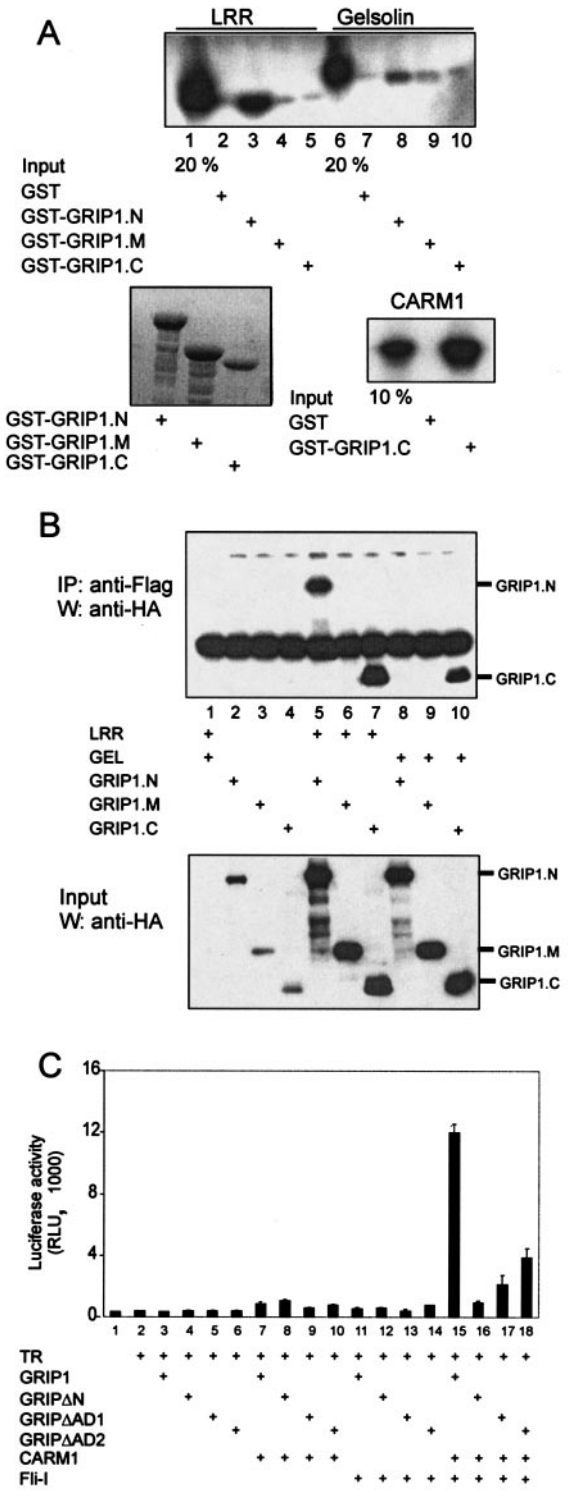

FIG. 6. Coactivator activity of Fli-I depends on the N-terminal domain of GRIP1. (A) LRR domain of Fli-I binds to GRIP1 N-terminal region in vitro. In vitro binding assays were performed as done for Fig. 1B. Upper panel tests the binding of ^{35}S-labeled Fli-I LRR and gelsolin-like fragments to GST and GST fused to GRIP1 N-terminal (GRIP1.N, amino acids 5 to 765), middle (GRIP1.M, amino acids 730 to 1121), and C-terminal (GRIP1.C, amino acids 1122 to 1462) fragments. Lower right panel shows binding of ^{35}S-labeled CARM1 to GST-GRIP1C. Lower left panel shows Coomassie-stained gel of the GST-GRIP1 fusion proteins used. (B) Binding of Fli-I fragments to GRIP1 fragments in vivo. Coimmunoprecipitations were performed as done for Fig. 1C. Cos-7 cells were transfected with pSG5.Flag-FliI(LRR) or pSG5.Flag-FliI(gelsolin) (3 μg) and pSG5.HA plasmid encoding GRIP1N, GRIP1 M, or GRIP1C (3 μg). Immunoprecipitation (IP) was performed with anti-Flag antibodies, and immunoblotting (W) was performed with anti-HA antibodies. Immunoblotting of immunoprecipitated proteins is shown in the upper panel, and immunoblotting of the cell extracts before immunoprecipitation is shown in the lower panel. (C) Requirement for GRIP1 N-terminal region in coactivator synergy. CV-1 cells were transiently transfected with MMTV(TRE)-LUC (250 ng) and expression vectors encoding TR (1 ng), CARM1 (500 ng), Fli-I (500 ng), and the wild type or indicated mutant of GRIP1 (250 ng): GRIP1ΔN lacked amino acids 1 to 562, ΔAD1 lacked amino acids 1057 to 1109, and ΔAD2 lacked amino acids 1122 to 1462. Transfected cells were grown with T3 before harvest. Results are representative of two independent experiments.

(ΔAD1, lacking amino acids 1057 to 1109), or the AD2 domain (ΔAD2, lacking amino acids 1122 to 1462) were coexpressed with CARM1, Fli-I, and low levels of TR in transient-transfection assays. As shown above (Fig. 5), reporter gene activation by hormone-activated TR under these conditions was synergistically enhanced by wild-type GRIP1, CARM1, and Fli-I, and all three coactivators were required for efficient reporter gene expression (Fig. 6C, assays 1 to 15). In contrast, the GRIP1ΔN mutant had no activity when coexpressed with CARM1 and Fli-I (assay 16); the GRIP1ΔAD1 and GRIP1ΔAD2 mutants exhibited weak partial activity in cooperation with CARM1 and Fli-I (assays 17 and 18). All three mutants were expressed at levels equal to or exceeding that of wild-type GRIP1 (data not shown) (42). Thus, the GRIP1 N-terminal domain, which is the direct binding site for Fli-I, was absolutely required for GRIP1-CARM1-Fli-I coactivator synergy. The strong requirement for the N-terminal domain, which is not essential for the binding of GRIP1 to NR, p300, or CARM1 (11, 29, 42), suggests that the interaction between the GRIP1 N-terminal domain and Fli-I is important for the recruitment of Fli-I to the coactivator complex and for the functional synergy between GRIP1 and Fli-I. The partial requirement for AD1 suggests that endogenous p300 or CBP, which binds to AD1, may contribute to the GRIP1–CARM1–Fli-I synergy. Although the AD2 domain was important for achieving full GRIP1–CARM1–Fli-I synergy, the fact that it was not absolutely required was somewhat surprising, since CARM1 binds to the AD2 region of GRIP1; the fact that CARM1 can bind to Fli-I may provide an alternative mechanism for recruiting CARM1 to the coactivator complex and thus for achieving the partial coactivator synergy observed with the GRIP1ΔAD2 mutant. This interpretation is supported by the fact that the AD2 domain is absolutely required for synergy between GRIP1, CARM1, and p300 (35); i.e., since Fli-I was not included in that case, there was no alternative binding site for CARM1.

siRNAs for Fli-I suppress ER-mediated transcription. To further test the physiological relevance of Fli-I as a coactivator, siRNAs directed toward the LRR domain of Fli-I were designed and tested for effects on ER transcription. The specificity of these siRNAs was tested by cotransfecting them into Cos-7 cells with separate vectors encoding the Flag-tagged LRR and gelsolin-like domains of Fli-I. In an immunoblot analysis of the transfected-cell extracts with anti-Flag antibody,

the two siRNAs for Fli-I completely or partially inhibited the expression of the LRR domain (Fig. 7A, lanes 2 to 4), whereas siRNAs for AIB1 and CARM1 did not influence the expression of LRR (lanes 5 and 6). In contrast, the two siRNAs for Fli-I had no effect on the expression of the Fli-I gelsolin-like domain (lanes 8 and 9), confirming the specificity of the Fli-I siRNAs.

Next, the effects of the same siRNAs on endogenous proteins were investigated by transfecting them into MCF-7 cells and by monitoring the expression levels of endogenous proteins by immunoblot. Endogenous Fli-I protein was specifically reduced by the two siRNAs for Fli-I but not by siRNAs for AIB1 or CARM1 (Fig. 7B, top panel). Endogenous CARM1 expression was inhibited by siRNA for CARM1 but not by the other siRNAs (middle panel). The endogenous actin level was not changed by any of the siRNAs. Thus, the siRNAs tested here are efficient and specific in reducing endogenous protein expression.

The effect of these siRNAs on transcriptional activation by endogenous ER was investigated in MCF-7 cells. MCF-7 cells were transfected with an estrogen-responsive reporter plasmid, and the transcription activation by endogenous ER was determined by reporter analysis. The estrogen-responsive reporter gene activation by endogenous ER was significantly enhanced by E2 (Fig. 7C, assays 1 and 6). Cotransfection of the reporter plasmid together with either Fli-I siRNA, the CARM1 siRNA, or the AIB1 siRNA resulted in loss of most of the estrogen-dependent reporter gene expression (assays 6 to 14), indicating that endogenous AIB1, CARM1, and Fli-I were all required for endogenous ER function. The siRNAs for Fli-I were reproducibly more efficient in inhibiting ER activity than were the CARM1 and AIB1 siRNAs (data not shown). siRNA directed against an irrelevant sequence had no effect on reporter gene activation by E2 and endogenous ER (data not shown). These data demonstrate the importance of Fli-I as a coactivator for the action of endogenous ER.

Role of actin binding activity of Fli-I gelsolin-like domains in coactivator function. Because the gelsolin-like domain of Fli-I is important for Fli-I coactivator function (Fig. 5A) and also can bind to actin (16, 17, 26, 39), we questioned whether the actin binding activity of Fli-I may be important for its coactivator function. In fact, some actin binding proteins, including supervillin and gelsolin itself, have already been shown to bind to and enhance transcriptional activation by NRs (28, 59). Actin and actin-like proteins are components of a number of nuclear complexes involved in transcription and its regulation, including the SWI/SNF ATP-dependent chromatin-remodeling complex (4, 52, 53) (see Discussion). Like the protein gelsolin, the gelsolin-like domain of Fli-I consists of two gelsolin-like motifs, each of which contains three repeats of an actin binding motif (8). In coimmunoprecipitation assays, we found that the first of the two gelsolin-like motifs in Fli-I (Gel.A, amino acids 495 to 827) can bind to Brg- or Brm-associated factor 53 (BAF53), an actin-like component of the SWI/SNF complex (Fig. 8A, upper panel). We next tested whether expression of the Gel.A fragment of Fli-I would either enhance (i.e., serve as a coactivator for) or inhibit (i.e., act as a dominant-negative mutant to interfere with the action of) ER function. Coexpression of the Gel.A fragment of Fli-I with ER in transient-transfection assays enhanced estrogen-dependent

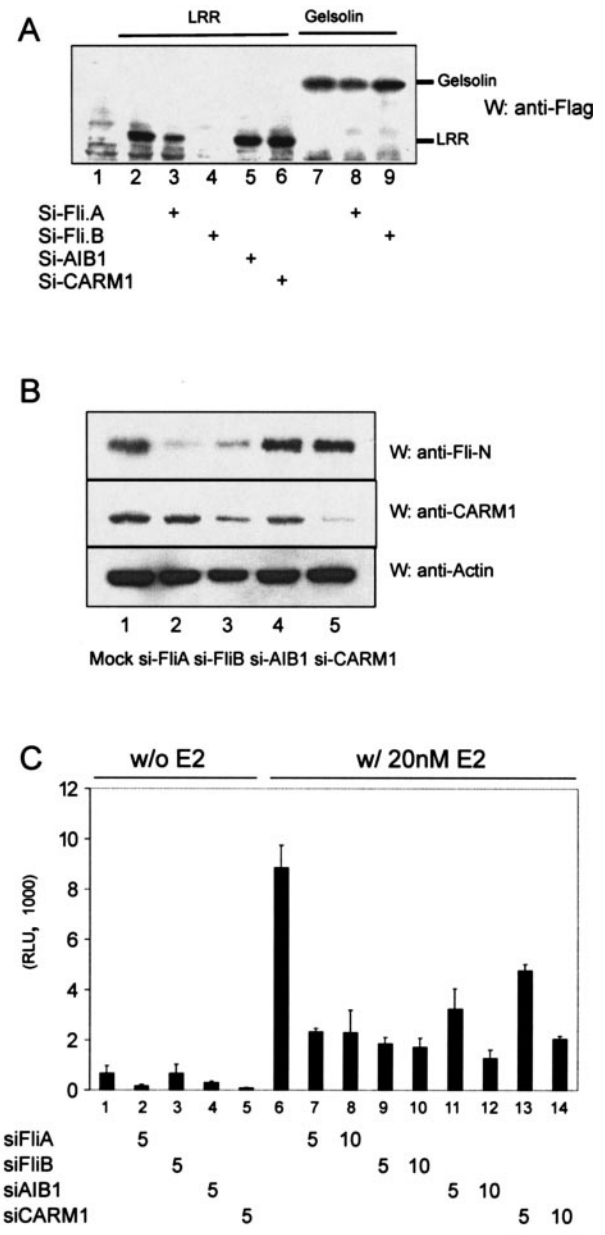

FIG. 7. Requirement for Fli-I in MCF-7 cells for ER-dependent gene expression. (A) Specificity of LRR-directed siRNAs. Cos-7 cells (in 12-well culture dishes with 1 ml of medium per well) were transfected with a pSG5.Flag vector encoding the LRR or gelsolin-like domain of Fli-I (1 μg) along with 5 μl of 20 μM siRNAs. Forty hours after transfection, cell extracts were analyzed by immunoblotting (W) with anti-Flag antibody. Lane 1 shows extract from cells transfected with empty vector and no siRNA. (B) Reduction of endogenous Fli-I protein level in MCF-7 cells by siRNAs for Fli-I. The indicated siRNAs were transfected as done for panel A into MCF-7 cells, and endogenous protein expression was analyzed by immunoblotting (W) with the antibody indicated on the right. (C) siRNAs for Fli-I suppress gene activation by endogenous ER and coactivators in MCF-7 cells. MMTV(ERE)-LUC reporter plasmid (1 μg) was transfected into MCF-7 cells in 12-well culture dishes containing 1 ml of medium along with the indicated siRNAs (5 or 10 μl of 20 μM siRNA). Before transfection, MCF-7 cells were maintained in phenol red-free Dulbecco's modified Eagle medium with 5% charcoal-dextran-treated fetal bovine serum for at least 4 days. After transfection of siRNAs, MCF-7 cells were treated with 20 nM E2 as indicated. Luciferase activity was determined 48 h after transfection. The data shown are representative of 10 independent experiments.

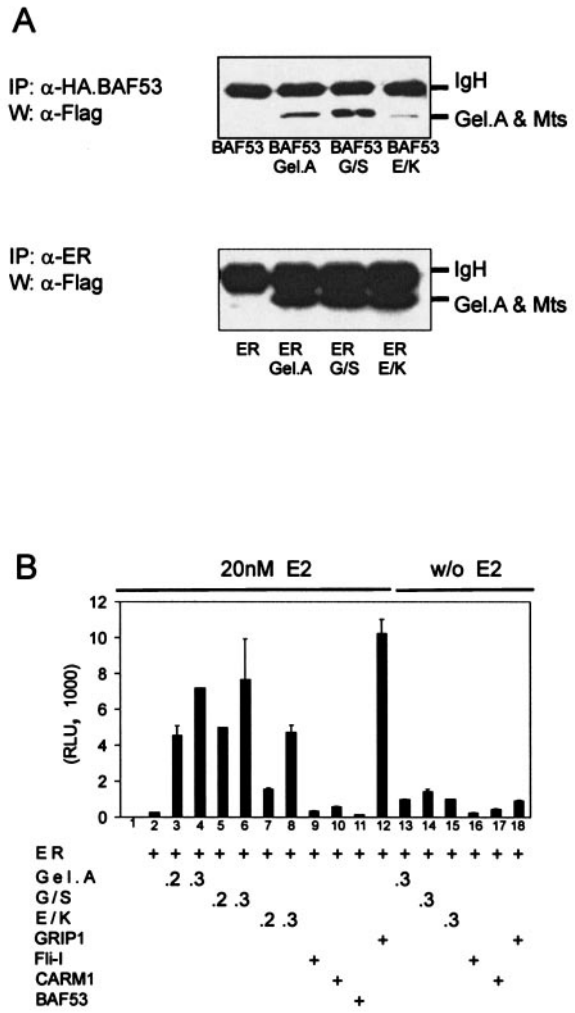

FIG. 8. Role of actin binding activity of Fli-I gelsolin-like domains in coactivator function. (A) Binding of Fli-I Gel.A domain (wild type and mutants) to actin-like molecule BAF53 and to ER. Vectors encoding the human Fli-I gelsolin-like Gel.A fragment (amino acids 495 to 827) and two point mutants (E585K and G602S) (2.5 μg) were transfected into Cos-7 cells along with vectors encoding the actin-like protein BAF53 (2.5 μg, upper panel) or ER (2.5 μg, lower panel). Cell lysates were prepared and immunoprecipitated (IP) with antibodies against HA or ER. The precipitated fractions were analyzed by immunoblotting (W) with anti-Flag antibody. (B) Coactivator activity of wild-type and mutant Fli-I Gel.A fragments. pSG5.Flag vectors encoding wild-type or mutant Fli-I Gel.A fragments (0.5 μg) were transfected into CV1 cells with 0.1 μg of ER expression vector and 0.25 μg of MMTV(ERE)-LUC reporter plasmid; E2 was added after transfection, and luciferase activity was determined 40 h after transfection. The data shown are representative of seven independent experiments.

expression of a transient reporter gene (Fig. 8B, assays 1 to 4 and 13).

In previous mutational studies with gelsolin, the first putative actin binding repeat (of three) within the first gelsolin-like motif (of two) was found to be more important for binding G-actin, while the second and third repeats in the first motif were shown to be more important for binding F-actin (8). The Gel.A fragment of Fli-I represents the first of the two gelsolin-like motifs in Fli-I. To test the importance of actin binding activity in the coactivator function of the Gel.A fragment of Fli-I, two point mutations were generated in the first of the three putative actin binding repeats within Gel.A. The E585K mutant of Gel.A was designed according to a previously reported mutation in the gelsolin protein that is defective in actin binding (64); the G602S mutation of Gel.A corresponds to the *Drosophila fliI*[3] mutation, which causes a partial defect in *Drosophila* muscle development and results in the flightless phenotype (18). In coimmunoprecipitation assays, the G/S mutation had no effect on binding of Gel.A to BAF53, but the E/K mutation reduced Gel.A binding to BAF53 (Fig. 8A, upper panel). In contrast, both mutants retained the ability of the wild-type Gel.A fragment to bind ER (Fig. 8A, lower panel); the retention of ER-binding activity shows that both mutants are folded properly and that the surfaces of Gel.A that interact with actin and with ER are distinct.

The Gel.A wild-type and mutant proteins were compared for their ability to serve as coactivators for ER. The G/S mutant was equivalent to the wild-type Gel.A fragment in its ability to enhance estrogen-dependent activation of a transiently transfected reporter gene by ER; however, the coactivator function of the E/K mutant was substantially reduced (Fig. 8B). The reduced activity of the E/K mutant was not due to improper folding or diminished expression or stability, as shown by the ability of this mutant to bind ER normally (Fig. 8A). The specific, correlated loss of actin binding (64) and of binding to actin-like BAF53 with loss of coactivator function in Gel.A mutants suggests that binding of actin or actin-like proteins is important for the coactivator function of the Gel.A fragment and, by extension, the coactivator function of Fli-I.

DISCUSSION

Fli-I is a novel secondary coactivator for NRs. Fli-I can function in cooperation with specific combinations of other NR coactivators to enhance the ability of NRs to activate transcription of NR-regulated reporter genes (Fig. 4 and 5). Fli-I was identified because of its binding to CARM1 (Fig. 1), but it also binds specifically in vitro and in vivo to GRIP1 (Fig. 6) and to at least two NRs (Fig. 2). Which of these protein-protein interactions is important for the coactivator function of Fli-I? Since Fli-I by itself did not enhance the activity of NRs but required the presence of a p160 coactivator (Fig. 4 and 5), the binding of Fli-I to NRs is apparently not sufficient for Fli-I coactivator function. However, the ability of ER to enhance the autonomous activation function of the Fli-I LRR domain (Fig. 4D) and the ability of the gelsolin-like Gel.A fragment of Fli-I to enhance ER function (Fig. 8B) indicate the existence of a functional relevance to the ER-Fli-I interaction and thus suggest that the Fli-I-NR interaction may contribute to the ability of Fli-I to serve as an NR coactivator.

Enhancement of NR function by Fli-I was observed only when GRIP1 was coexpressed with Fli-I, whether or not additional coactivators such as CARM1 and p300 were also coexpressed (Fig. 4 and 5). The dependence of Fli-I coactivator function on GRIP1 indicates that Fli-I is a secondary coactivator and therefore that the binding of Fli-I to GRIP1 is essential for the coactivator function of Fli-I. While Fli-I bound to both the N-terminal and C-terminal regions of GRIP1 in coimmunoprecipitation experiments (Fig. 6B), Fli-I

bound only to the N-terminal region of GRIP1 in vitro (Fig. 6A), suggesting a direct interaction with the N-terminal region of GRIP1 and an indirect binding to the C-terminal region of GRIP1. Furthermore, the cooperative coactivator activity of Fli-I with CARM1 and GRIP1 was absolutely dependent on the N-terminal region of GRIP1 but was only partially dependent on the C-terminal AD1 and AD2 regions of GRIP1 (Fig. 6C). Thus, the binding of Fli-I to the N-terminal region of GRIP1 appears to be the critical binding interaction that potentiates Fli-I recruitment to the target gene promoter. While we have no definitive data at present as to whether the Fli-I-CARM1 binding is necessary for Fli-I coactivator function, the functional synergy between these two coactivators (Fig. 5) suggests that this binding interaction may well contribute. The contacts between Fli-I and multiple components in the transcription complex (NRs, GRIP1, and CARM1) may result in a more efficient recruitment of Fli-I to the promoter, a more stable coactivator complex, or a more highly functional conformation of the coactivator complex.

Both the LRR and gelsolin-like domains of Fli-I were required for its coactivator function. While the LRR domain alone had modest coactivator function in cooperation with GRIP1 at high levels of NRs (Fig. 4C), both Fli-I domains were required absolutely under low-NR conditions (Fig. 5A). The binding studies suggest that the LRR domain is primarily or solely responsible for the critical Fli-I-GRIP1.N interaction (Fig. 6). It appears that the binding of Fli-I to CARM1 and NRs also contributes to the cooperative coactivator function of Fli-I and that both the LRR and gelsolin-like domains participate in these binding interactions. The autonomous transcriptional activation activity in the LRR domain may explain why this domain exhibited modest coactivator function with GRIP1 under high-NR conditions and also suggests that this activation function may play a role in Fli-I coactivator activity, presumably through protein-protein interactions with other components of the transcription machinery.

Two other proteins previously found to bind to the Fli-I LRR domain include FLAP1 (also called LRRFIP2) and FLAP2 (also called TRIP, LRRFIP1, and GCF2) (9). Evidence to date suggests that FLAP2 can bind specific RNA sequences but can also function as a specific DNA-binding transcriptional repressor. Binding of Fli-I to the transcriptional regulator FLAP2 is interesting in light of, and also consistent with, our conclusions that Fli-I can function as a transcriptional coregulator for NRs. Separate studies found that the LRR domain of the *Caenorhabditis elegans* Fli-I homologue can bind to Ras but not to other small GTPases (9). While a similar binding interaction in higher organisms has yet to be confirmed, the interaction in *C. elegans* has interesting implications for possible regulation of Fli-I coactivator activity by signaling pathways involving specific small GTPases.

Characterization of CARM1 and GRIP1 function by identification of interacting proteins. The mechanisms by which CARM1 and GRIP1 enhance NR function involve the ability of CARM1 and GRIP1 to bind other proteins and transmit the transcriptional activation signal to them or cooperate with them as coactivators (10, 35, 42, 58). Thus, our data indicate that the coactivator function of CARM1 may involve its ability to bind to and function synergistically with Fli-I. The synergistic cooperation between CARM1 and Fli-I was specific, since Fli-I did not function synergistically with the protein acetyltransferase p300 or with PRMT1 (Fig. 5B and C), another member of the protein arginine methyltransferase family that methylates different protein substrates from CARM1 (57). The ability of Fli-I to bind strongly to CARM1 but only weakly to PRMT1 suggests that the binding between CARM1 and Fli-I contributes to the selective functional synergy observed between Fli-I and CARM1 but not PRMT1. The synergy between CARM1 and Fli-I required the methyltransferase activity of CARM1 (Fig. 5C). The target protein(s) that must be methylated by CARM1 to achieve coactivator synergy could include histone H3, which is known to be methylated by CARM1 during steroid hormone activation of stably integrated genes (41), and/or another presently unknown target, such as a component of the transcription machinery or another coactivator. While both CARM1 and Fli-I are apparently recruited to the promoter through their interaction with GRIP1, CARM1 binds to the C-terminal AD2 domain of GRIP1 (10, 11), whereas Fli-I binds to the N-terminal region of GRIP1 (Fig. 6). This observation and the synergistic cooperation between CARM1 and Fli-I indicate that these two coactivators have distinct but complementary roles in the transcriptional activation process. That is, they mediate the functions of two different regions of GRIP1 and also contribute to the transcriptional activation process by two different mechanisms. CARM1 methylates histones, p300/CBP, and possibly other proteins in the transcription initiation complex (41, 69) and also may recruit additional coactivators (58). The complementary role of Fli-I could involve a specific protein (presently unknown) that interacts with and is the target of the autonomous transcriptional activation domain in the LRR region of Fli-I; in addition, the ability of the Fli-I gelsolin-like domain to bind actin (16, 17) and actin-like proteins may be important for the coactivator function of Fli-I (Fig. 8; also see additional discussion below).

The cooperation of Fli-I with GRIP1 also provides additional insight into the mechanism of coactivator function by GRIP1. The N-terminal 350 amino acids of GRIP1 and the other two members of the p160 coactivator family is the most highly conserved region of the family (2), but the role of this region in p160 coactivator function is unclear. One basic helix-loop-helix (bHLH) and two Per-Arnt-Sim (PAS) motifs are found in this region. Recently, several proteins that bind to this region of p160 coactivators have been identified. Some of these proteins, including TEF4 (5) and MEF2C (14), are DNA-binding transcriptional activators, which apparently recruit p160 coactivators by binding to the bHLH-PAS region; thus p160 coactivators can function as coactivators for many different classes of transcriptional activator proteins in addition to NRs. Other coactivators, including BAF57 (4) and MMS19 (66), also bind to the bHLH-PAS region of p160 coactivators. While these findings suggest that the N-terminal bHLH-PAS region may contribute to p160 coactivator function by recruiting additional coactivators to the promoter, a clear demonstration of this relationship has not previously been shown. The fact that Fli-I binds to the N-terminal region of GRIP1 and cooperates synergistically with GRIP1 as a coactivator demonstrates a functional relevance for the binding of Fli-I to GRIP1 and indicates that one role of the N-terminal region of GRIP1 is to recruit Fli-I as a coactivator, just as the AD1 domain of

p160 coactivators recruits p300 or CBP and just as the AD2 domain of p160 coactivators recruits CARM1. Thus, our results demonstrate that the N-terminal region of p160 coactivators can serve as an additional activation or signal transmission domain, along with AD1 and AD2.

The p160 coactivators appear to serve as scaffold proteins that contact NRs, as well as other classes of DNA-binding transcriptional activator proteins, directly and appear to assemble a complex of other coactivators on the promoter (25, 46). The specific complement of coactivators required may vary for different promoters and in different cell types. For example, our results show that different domains of GRIP1 are important to potentiate synergy between different combinations of coactivators. When GRIP1 acts synergistically with CARM1 and p300, the AD2 domain is absolutely required, while deletion of the AD1 domain causes only partial loss of synergy (35). In contrast, when GRIP1 acts synergistically with CARM1 and Fli-I, the AD1 and AD2 domains both contribute but are not absolutely required (Fig. 6C). Thus, the specific domains of p160 coactivators required may vary depending on what complex of secondary coactivators is available and/or assembled on the promoter. In addition, since AD2 is the direct binding site for CARM1, the reduced requirement for the AD2 domain when Fli-I is present with CARM1 suggests that CARM1 can be recruited to the p160 coactivator indirectly by binding to Fli-I, which is bound to the N-terminal region of the p160 coactivator.

Implications of the actin binding properties of Fli-I for its coactivator function. The gelsolin-like motifs of Fli-I can bind to actin in both the G (globular) and F (polymerized) forms, and Fli-I was observed by immunofluorescence to associate with the actin cytoskeleton under certain cellular conditions (16, 17). The association with actin and the subcellular localization of Fli-I appear to be regulated by pathways involving phosphatidylinositol 3-kinase and Ras-related small GTPases; for example, in cells grown in low serum concentrations Fli-I was primarily nuclear, but at higher serum concentrations, Fli-I was found in the cytoplasmic compartment associated with the cytoskeleton (17). These observations are consistent with regulated dual roles for Fli-I as a transcriptional coactivator in the nucleus and a cytoplasmic modulator of the cytoskeleton; furthermore, they suggest that Fli-I may function as a communication link through which cytoskeleton structure may influence transcription.

In addition to its role as a cytoskeleton structural protein, previous studies suggested roles for actin in the transcription process (21, 54). Actin and actin-like proteins are found in the nucleus in association with various complexes associated with the transcription machinery (1). Both actin and the actin-like protein BAF53 were identified as integral and essential components of SWI/SNF coactivator complexes, which occur in multiple forms and contribute to the chromatin-remodeling process via an ATP-dependent nucleosome-remodeling activity (53, 70, 72). Another component of the SWI/SNF complexes, BAF57, can (like Fli-I) bind to both ER and the N-terminal region of p160 coactivators (4); the interaction between BAF57 and the ER-assembled transcription initiation complex suggests a possible mechanism for coordinating the actions of the SWI/SNF and p160 coactivator complexes, which remodel chromatin by different but complementary mechanisms, i.e., ATP-dependent nucleosome remodeling and histone modifications, respectively (25, 46). Overexpression of BAF57 was shown to enhance NR function in a cell line with low levels of endogenous BAF57 (4), but whether the p160-binding ability of BAF57 is important for the coactivator functions of BAF57 and the SWI/SNF complexes is not yet determined.

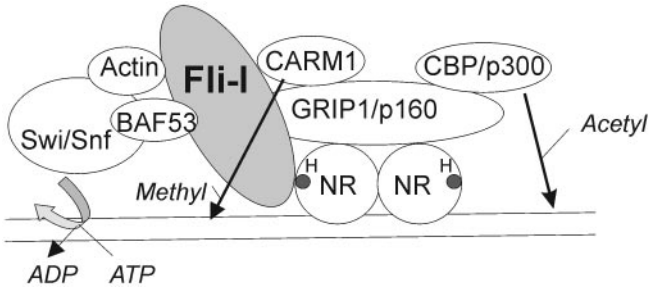

FIG. 9. Proposed model for cooperative linking of p160 and SWI/SNF coactivator complexes by Fli-I protein. Fli-I protein can bind to components of the p160 coactivator complex (p160 and CARM1), which contains histone acetylating (CBP/p300) and methylating (CARM1) activities. Fli-I can also bind to actin and the actin-like protein BAF53, both of which are components of the ATP-dependent nucleosome-remodeling complex SWI/SNF. Thus, one possible mechanism for the contribution of Fli-I as a coactivator could be to facilitate the recruitment of or somehow coordinate the complementary chromatin-remodeling activities of the SWI/SNF complex and the p160 coactivator complex. H, hormone.

A role for unknown actin binding proteins in the regulated recruitment of SWI/SNF complexes to chromatin was previously proposed (72). We have observed that Fli-I not only binds actin (17) but also binds the actin-like BAF53 in coimmunoprecipitation experiments (Fig. 8A), as well as p160 coactivators (Fig. 6). Thus, Fli-I, like BAF57, could help to secure the association of an SWI/SNF complex to a p160 coactivator complex and thus help to coordinate the complementary ATP-dependent nucleosome-remodeling activity of the SWI/SNF complex with the histone acetylating (e.g., from CBP and p300) and methylating (e.g., from CARM1 and PRMT1) activities of the p160 coactivator complex (Fig. 9).

Recently, another actin binding protein, supervillin, was reported as a coactivator for some NRs (59). The actin binding motif of supervillin, like that of Fli-I, is highly homologous to gelsolin. In addition, our yeast two-hybrid screen for CARM1 binding proteins identified not only Fli-I but also profilin 1 (data not shown), another actin binding protein (72). Although a possible role for profilin 1 as a coactivator remains to be tested, we speculate that Fli-I, supervillin, and profilin 1 may represent a class of actin binding coactivators with similar mechanisms of enhancing transcriptional activation by association with actin and actin-like proteins. The dual roles of Fli-I as an actin cytoskeleton-associated protein and transcriptional coactivator are not unprecedented. Another developmentally important protein, β-catenin, participates in regulation of the cytoskeleton by association with cell adhesion molecules (47) and also functions as a coactivator in the nucleus (20, 28, 61), where it can bind to and function synergistically with CARM1 as coactivators for NRs and other classes of DNA-binding transcriptional activator proteins (33). As is true for Fli-I, the

localization of β-catenin between cytoplasm and nucleus is regulated by specific signal transduction pathways. Thus, Fli-I may be part of another signal transduction pathway that links cytoskeleton structure with transcriptional regulation. Since CARM1 and Fli-I have a functional synergy as transcriptional coactivators, it is also tempting to speculate that the CARM1-Fli-I interaction could influence the cytoplasmic function of Fli-I as well as its nuclear activity.

ACKNOWLEDGMENTS

We thank Malcolm Parker (Imperial College Faculty of Medicine, London, United Kingdom) for plasmid pSG5.Flag-TEF4; Jeonghyun Park and Michael Cole (Princeton University, Princeton, N.J.) for the BAF53 expression vector; and Paul Webb and Peter Kushner (University of California, San Francisco) for the GST-ER expression vector pGEX5X-1.HEG0.

This work was supported by grant DK43093 to M.R.S. from the National Institutes of Health.

REFERENCES

1. **Ankenbauer, T., J. A. Kleinschmidt, M. J. Walsh, O. H. Weiner, and W. W. Franke.** 1989. Identification of a widespread nuclear actin binding protein. Nature **342:**822–825.
2. **Anzick, S. L., J. Kononen, R. L. Walker, D. O. Azorsa, M. M. Tanner, X.-Y. Guan, G. Sauter, O.-P. Kallioniemi, J. M. Trent, and P. S. Meltzer.** 1997. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science **277:**965–968.
3. **Beato, M., P. Herrlich, and G. Schütz.** 1995. Steroid hormone receptors: many actors in search of a plot. Cell **83:**851–857.
4. **Belandia, B., R. L. Orford, H. C. Hurst, and M. G. Parker.** 2002. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. EMBO J. **21:**4094–4103.
5. **Belandia, B., and M. G. Parker.** 2000. Functional interaction between the p160 coactivator proteins and the transcriptional enhancer factor family of transcription factors. J. Biol. Chem. **275:**30801–30805.
6. **Campbell, H. D., S. Fountain, I. S. McLennan, L. A. Berven, M. F. Crouch, D. A. Davy, J. A. Hooper, K. Waterford, K. S. Chen, J. R. Lupski, B. Ledermann, I. G. Young, and K. I. Matthaei.** 2002. Fliih, a gelsolin-related cytoskeletal regulator essential for early mammalian embryonic development. Mol. Cell. Biol. **22:**3518–3526.
7. **Campbell, H. D., S. Fountain, I. G. Young, S. Weitz, P. Lichter, and J. D. Hoheisel.** 2000. Fliih, the murine homologue of the Drosophila melanogaster flightless I gene: nucleotide sequence, chromosomal mapping and overlap with Llglh. DNA Seq. **11:**29–40.
8. **Campbell, H. D., T. Schimansky, C. Claudianos, N. Ozsarac, A. B. Kasprzak, J. N. Cotsell, I. G. Young, H. G. de Couet, and G. L. Miklos.** 1993. The Drosophila melanogaster flightless-I gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich repeat domains and is conserved in Caenorhabditis elegans and humans. Proc. Natl. Acad. Sci. USA **90:**11386–11390.
9. **Campbell, H. D., I. G. Young, and K. I. Matthaei.** 2000. Mammalian homologues of the Drosophila melanogaster flightless I gene involved in early development. Curr. Genom. **1:**59–70.
10. **Chen, D., S.-M. Huang, and M. R. Stallcup.** 2000. Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. J. Biol. Chem. **275:**40810–40816.
11. **Chen, D., H. Ma, H. Hong, S. S. Koh, S.-M. Huang, B. T. Schurter, D. W. Aswad, and M. R. Stallcup.** 1999. Regulation of transcription by a protein methyltransferase. Science **284:**2174–2177.
12. **Chen, H., R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M. L. Privalsky, Y. Nakatani, and R. M. Evans.** 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell **90:**569–580.
13. **Chen, K. S., P. H. Gunaratne, J. D. Hoheisel, I. G. Young, G. L. Miklos, F. Greenberg, L. G. Shaffer, H. D. Campbell, and J. R. Lupski.** 1995. The human homologue of the Drosophila melanogaster flightless-I gene (flil) maps within the Smith-Magenis microdeletion critical region in 17p11.2. Am. J. Hum. Genet. **56:**175–182.
14. **Chen, S. L., D. H. Dowhan, B. M. Hosking, and G. E. Muscat.** 2000. The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. Genes Dev. **14:**1209–1228.
15. **Darimont, B. D., R. L. Wagner, J. W. Apriletti, M. R. Stallcup, P. J. Kushner, J. D. Baxter, R. J. Fletterick, and K. R. Yamamoto.** 1998. Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. **12:**3343–3356.
16. **Davy, D. A., E. E. Ball, K. I. Matthaei, H. D. Campbell, and M. F. Crouch.** 2000. The flightless I protein localizes to actin-based structures during embryonic development. Immunol. Cell Biol. **78:**423–429.
17. **Davy, D. A., H. D. Campbell, S. Fountain, D. de Jong, and M. F. Crouch.** 2001. The flightless I protein colocalizes with actin- and microtubule-based structures in motile Swiss 3T3 fibroblasts: evidence for the involvement of PI 3-kinase and Ras-related small GTPases. J. Cell Sci. **114:**549–562.
18. **de Couet, H. G., K. S. Fong, A. G. Weeds, P. J. McLaughlin, and G. L. Miklos.** 1995. Molecular and mutational analysis of a gelsolin-family member encoded by the flightless I gene of Drosophila melanogaster. Genetics **141:**1049–1059.
19. **Ding, X. F., C. M. Anderson, H. Ma, H. Hong, R. M. Uht, P. J. Kushner, and M. R. Stallcup.** 1998. Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol. Endocrinol. **12:**302–313.
20. **Eastman, Q., and R. Grosschedl.** 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr. Opin. Cell Biol. **11:**233–240.
21. **Egly, J. M., N. G. Miyamoto, V. Moncollin, and P. Chambon.** 1984. Is actin a transcription initiation factor for RNA polymerase B? EMBO J. **3:**2363–2371.
22. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
23. **Frankel, A., N. Yadav, J. Lee, T. L. Branscombe, S. Clarke, and M. T. Bedford.** 2002. The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. J. Biol. Chem. **277:**3537–3543.
24. **Gehin, M., M. Mark, C. Dennefeld, A. Dierich, H. Gronemeyer, and P. Chambon.** 2002. The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. Mol. Cell. Biol. **22:**5923–5937.
25. **Glass, C. K., and M. G. Rosenfeld.** 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. **14:**121–141.
26. **Goshima, M., K. Kariya, Y. Yamawaki-Kataoka, T. Okada, M. Shibatohge, F. Shima, E. Fujimoto, and T. Kataoka.** 1999. Characterization of a novel Ras-binding protein Ce-FLI-1 comprising leucine-rich repeats and gelsolin-like domains. Biochem. Biophys. Res. Commun. **257:**111–116.
27. **Heery, D. M., E. Kalkhoven, S. Hoare, and M. G. Parker.** 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature **387:**733–736.
28. **Heinlein, C. A., and C. Chang.** 2002. Androgen receptor (AR) coregulators: an overview. Endocr. Rev. **23:**175–200.
29. **Hong, H., K. Kohli, A. Trivedi, D. L. Johnson, and M. R. Stallcup.** 1996. GRIP1, a novel mouse protein that serves as a transcriptional co-activator in yeast for the hormone binding domains of steroid receptors. Proc. Natl. Acad. Sci. USA **93:**4948–4952.
30. **Huang, S.-M., and M. R. Stallcup.** 2000. Mouse Zac1, a transcriptional coactivator and repressor for nuclear receptors. Mol. Cell. Biol. **20:**1855–1867.
31. **Kobe, B., and A. V. Kajava.** 2001. The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. **11:**725–732.
32. **Koh, S. S., D. Chen, Y.-H. Lee, and M. R. Stallcup.** 2001. Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. J. Biol. Chem. **276:**1089–1098.
33. **Koh, S. S., H. Li, Y. H. Lee, R. B. Widelitz, C. M. Chuong, and M. R. Stallcup.** 2002. Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. J. Biol. Chem. **277:**26031–26035.
34. **Kwiatkowski, D. J.** 1999. Functions of gelsolin: motility, signaling, apoptosis, cancer. Curr. Opin. Cell Biol. **11:**103–108.
35. **Lee, Y.-H., S. S. Koh, X. Zhang, X. Cheng, and M. R. Stallcup.** 2002. Synergy among nuclear receptor coactivators: selective requirement for protein methyltransferase and acetyltransferase activities. Mol. Cell. Biol. **22:**3621–3632.
36. **Li, H., S. Park, B. Kilburn, M. A. Jelinek, A. Henschen-Edman, D. W. Aswad, M. R. Stallcup, and I. A. Laird-Offringa.** 2002. Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. J. Biol. Chem. **277:**44623–44630.
37. **Li, J., B. W. O'Malley, and J. Wong.** 2000. p300 requires its histone acetyltransferase activity and SRC-1 interaction domain to facilitate thyroid hormone receptor activation in chromatin. Mol. Cell. Biol. **20:**2031–2042.
38. **Lin, W.-J., J. D. Gary, M. C. Yang, S. Clarke, and H. R. Herschman.** 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. J. Biol. Chem. **271:**15034–15044.
39. **Liu, Y. T., and H. L. Yin.** 1998. Identification of the binding partners for flightless I, a novel protein bridging the leucine-rich repeat and the gelsolin superfamilies. J. Biol. Chem. **273:**7920–7927.
40. **Liu, Z., J. Wong, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 2001. Sequential recruitment of steroid receptor coactivator-1 (SRC-1) and p300 enhances

progesterone receptor-dependent initiation and reinitiation of transcription from chromatin. Proc. Natl. Acad. Sci. USA **98:**12426–12431.

41. **Ma, H., C. T. Baumann, H. Li, B. D. Strahl, R. Rice, M. A. Jelinek, D. W. Aswad, C. D. Allis, G. L. Hager, and M. R. Stallcup.** 2001. Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on the mouse mammary tumor virus promoter. Curr. Biol. **11:**1981–1985.
42. **Ma, H., H. Hong, S.-M. Huang, R. A. Irvine, P. Webb, P. J. Kushner, G. A. Coetzee, and M. R. Stallcup.** 1999. Multiple signal input and output domains of the 160-kDa nuclear receptor coactivator proteins. Mol. Cell. Biol. **19:** 6164–6173.
43. **Ma, H., Y. Shang, D. Y. Lee, and M. R. Stallcup.** 2003. Study of nuclear receptor-induced transcription complex assembly and histone modification by chromatin immunoprecipitation. Methods Enzymol. **364:**284–296.
44. **Mangelsdorf, D. J., and R. M. Evans.** 1995. The RXR heterodimers and orphan receptors. Cell **83:**841–850.
45. **McBride, A. E., V. H. Weiss, H. K. Kim, J. M. Hogle, and P. A. Silver.** 2000. Analysis of the yeast arginine methyltransferase Hmt1p/Rmt1p and its in vivo function. Cofactor binding and substrate interactions. J. Biol. Chem. **275:**3128–3136.
46. **McKenna, N. J., and B. W. O'Malley.** 2002. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell **108:**465–474.
47. **Morin, P. J.** 1999. Beta-catenin signaling and cancer. Bioessays **21:**1021–1030.
48. **Nolte, R. T., G. B. Wisely, S. Westin, J. E. Cobb, M. H. Lambert, R. Kurokawa, M. G. Rosenfeld, T. M. Willson, C. K. Glass, and M. V. Milburn.** 1998. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature **395:**137–143.
49. **Oñate, S. A., S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley.** 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science **270:**1354–1357.
50. **Park, J., M. A. Wood, and M. D. Cole.** 2002. BAF53 forms distinct nuclear complexes and functions as a critical c-Myc-interacting nuclear cofactor for oncogenic transformation. Mol. Cell. Biol. **22:**1307–1316.
51. **Puri, P. L., V. Sartorelli, X. J. Yang, Y. Hamamori, V. V. Ogryzko, B. H. Howard, L. Kedes, J. Y. Wang, A. Graessmann, Y. Nakatani, and M. Levrero.** 1997. Differential roles of p300 and PCAF acetyltransferases in muscle differentiation. Mol. Cell **1:**35–45.
52. **Rando, O. J., K. Zhao, and G. R. Crabtree.** 2000. Searching for a function for nuclear actin. Trends Cell Biol. **10:**92–97.
53. **Rando, O. J., K. Zhao, P. Janmey, and G. R. Crabtree.** 2002. Phosphatidylinositol-dependent actin filament binding by the SWI/SNF-like BAF chromatin remodeling complex. Proc. Natl. Acad. Sci. USA **99:**2824–2829.
54. **Scheer, U., H. Hinssen, W. W. Franke, and B. M. Jockusch.** 1984. Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes. Cell **39:**111–122.
55. **Shang, Y., and M. Brown.** 2002. Molecular determinants for the tissue specificity of SERMs. Science **295:**2465–2468.
56. **Shang, Y., X. Hu, J. DiRenzo, M. A. Lazar, and M. Brown.** 2000. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell **103:**843–852.
57. **Stallcup, M. R.** 2001. Role of protein methylation in chromatin remodeling and transcriptional regulation. Oncogene **20:**3014–3020.
58. **Teyssier, C., D. Chen, and M. R. Stallcup.** 2002. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. J. Biol. Chem. **277:**46066–46072.
59. **Ting, H. J., S. Yeh, K. Nishimura, and C. Chang.** 2002. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc. Natl. Acad. Sci. USA **99:**661–666.
60. **Torchia, J., D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass, and M. G. Rosenfeld.** 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear receptor function. Nature **387:**677–684.
61. **Truica, C. I., S. Byers, and E. P. Gelmann.** 2000. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res. **60:**4709–4713.
62. **Tsai, M.-J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63:** 451–486.
63. **Voegel, J. J., M. J. S. Heine, M. Tini, V. Vivat, P. Chambon, and H. Gronemeyer.** 1998. The coactivator TIF2 contains three nuclear receptor binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. EMBO J. **17:**507–519.
64. **Way, M., B. Pope, and A. G. Weeds.** 1992. Are the conserved sequences in segment 1 of gelsolin important for binding actin? J. Cell Biol. **116:**1135–1143.
65. **Webb, P., G. N. Lopez, R. M. Uht, and P. J. Kushner.** 1995. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol. Endocrinol. **9:**443–456.
66. **Wu, X., H. Li, and J. D. Chen.** 2001. The human homologue of the yeast DNA repair and TFIIH regulator MMS19 is an AF-1-specific coactivator of estrogen receptor. J. Biol. Chem. **276:**23962–23968.
67. **Xu, J., L. Liao, G. Ning, H. Yoshida-Komiya, C. Deng, and B. W. O'Malley.** 2000. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc. Natl. Acad. Sci. USA **97:**6379–6384.
68. **Xu, J., Y. Qiu, F. J. DeMayo, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 1998. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science **279:**1922–1925.
69. **Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B. M. Emerson, M. Montminy, and R. M. Evans.** 2001. A transcriptional switch mediated by cofactor methylation. Science **294:**2507–2511.
70. **Xue, Y., J. C. Canman, C. S. Lee, Z. Nie, D. Yang, G. T. Moreno, M. K. Young, E. D. Salmon, and W. Wang.** 2000. The human SWI/SNF-B chromatin-remodeling complex is related to yeast rsc and localizes at kinetochores of mitotic chromosomes. Proc. Natl. Acad. Sci. USA **97:**13015–13020.
71. **Zhang, X., L. Zhou, and X. Cheng.** 2000. Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. EMBO J. **19:**3509–3519.
72. **Zhao, K., W. Wang, O. J. Rando, Y. Xue, K. Swiderek, A. Kuo, and G. R. Crabtree.** 1998. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell **95:**625–636.