The enhancement of nuclear receptor transcriptional activation by a mouse actin-binding protein, alpha actinin 2

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Abstract

The p160 coactivators, steroid receptor coactivator 1, glucocorticoid receptor interacting protein 1 (GRIP1) and the activator of thyroid and retinoic acid receptor, have two activation domains, AD1 and AD2, which transmit the activation signal from the DNA-bound nuclear receptor to the chromatin and/or transcription machinery. In screening for mammalian proteins that bind the AD2 of GRIP1, we identified a mouse actin-binding protein, alpha actinin 2 (mACTN2). mACTN2 was expressed in the heart, skeletal muscle, lung, brain and testis, but there was no expression in the spleen, liver or kidney. Interestingly, the expression level of mACTN2 in the developing embryo depended on the embryonic stage. We further demonstrated that mACTN2 could enhance two transactivation activities of GRIP1, which in turn could enhance the homodimerization of mACTN2. Importantly, mACTN2 not only served as a primary coactivator for androgen receptor, estrogen receptor and thyroid receptor activities, but also acted synergistically with GRIP1 to enhance these nuclear receptor (NR) functions. However, the NR binding motif, LXXLL, conserved in mACTN2 and other actinin family proteins, might be a dispensable domain for its coactivator roles in NRs. These findings suggested that mACTN2 might play an important role in GRIP1-induced NR coactivator functions.

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Introduction

Nuclear receptors (NRs) make up a family of transcription factors that regulate gene expression in a ligand-dependent manner (Tsai & O'Malley 1994, Beato *et al.* 1995, Mangelsdorf & Evan 1995). Members include the receptors for steroid, thyroid, retinoid and vitamin D hormones. In addition, some unidentified regulatory ligands bind to so-called orphan receptors of the NR superfamily. A highly conserved DNA-binding domain (DBD) located in the central portion of the polypeptide chain is the hallmark of all NR structures. The hormone-binding domain, which is conserved to a lesser extent than the DBD, is a large C-terminal domain. In addition to binding hormone, the hormone-binding domain also contains an important and highly conserved activation function, AF-2,

which is one of the two domains primarily responsible for the activation of transcription by hormone-activated DNA-bound NR. The other activation function, AF-1, found in the N-terminal domain of most NRs, is not conserved in length or sequence. The relative importance of AF-1 and AF-2 varies among different NRs and can be influenced by ligand, cell type and target gene promoter. Most ligands that bind NRs as homo- or heterodimers activate transcription by binding to enhancer elements in the promoters of target genes. However, some members of the NR family regulate transcription by binding to other classes of DNA-bound transcription factors. In addition to gene activation, a subset of NRs, including thyroid receptor (TR) and retinoic acid receptor, can actively repress target genes in the presence or absence of ligand binding, while many NRs inhibit

transcription in a ligand-dependent manner by antagonizing the transcriptional activities of other classes of transcription factors. The mechanism of transcriptional regulation by the DNA-bound NRs appears to involve their ability to recruit a variety of coregulatory proteins, including coactivators and corepressors (Glass & Rosenfeld 2000). Generally, coactivators are not DNA-binding proteins, but rather are recruited to the promoter through protein–protein interaction with the transcriptional activators. They may be thought of as adaptors or components in a signaling pathway that transmits transcriptional activation signals from DNA-bound activator proteins to the chromatin and transcription machinery. Transcriptional corepressors could involve competition for limiting factors, displacement of positive factors, or the generation of a chromatin structure that limits promoter accessibility by histone deacetylation. Therefore, the latest working model of NRs is an initial association with transcriptional corepressors and subsequent ability to recruit coactivators in response to ligands and other signals (Feng *et al.* 1998).

NR coactivator proteins, recruited by ligand-bound NRs or their activation functions, modify local chromatin structure by catalyzing covalent histone modifications and directly assembling and/or stabilizing the transcription preinitiation complex. A growing list of putative NR coactivators has been identified by their ability to bind and/or enhance the activity of NRs. Three major protein families function in a coactivator complex associated with the DNA-bound NRs: the p160 coactivators, cAMP-responsive element-binding protein-binding protein (CBP)/p300, and p300/CBP-associated factor (p/CAF) (Freedman 1999, McKenna *et al.* 1999, Glass & Rosenfeld 2000). The p160 coactivators include steroid receptor coactivator 1, glucocorticoid receptor interacting protein 1 (GRIP1) and the activator of thyroid and retinoic acid receptor. They bind directly to the hormone-binding domain of NRs by their LXXLL motifs, where L is leucine and X is any amino acid, and enhance NR AF-2 activity in the presence of appropriate ligands (Heery *et al.* 1997, Feng *et al.* 1998, McInerney *et al.* 1998). The p160 coactivators also bind the AF-1 region of some NRs, including progesterone receptor, estrogen receptor (ER), TR, mineralocorticoid receptor and androgen receptor (AR), and thereby enhance these AF-1 functions (Oñate *et al.* 1998, Webb *et al.*

1998, Bevan *et al.* 1999, Ma *et al.* 1999, Tremblay *et al.* 1999, Fuse *et al.* 2000, Oberste-Berghaus *et al.* 2000). CBP, p300 and p/CAF bind directly to the NR and to the p160 coactivators (Voegel *et al.* 1996, Yao *et al.* 1996, Chen *et al.* 1997, Torchia *et al.* 1997, Blanco *et al.* 1998, Ma *et al.* 1999). The histone acetyltransferase activity of these coactivators can acetylate histones, transcriptional activators and components of the transcription initiation complex, leading to the proposal that CBP and p300 serve as platforms to integrate the effects of multiple signaling pathways on many different transcriptional activator proteins (Chakravarti *et al.* 1996, Kamei *et al.* 1996, Ogryzko *et al.* 1996, Swope *et al.* 1996, Goodman & Smolik 2000, Strahl & Allis 2000).

The p160 coactivators have two activation domains, AD1 and AD2, which transmit the activating signal from the DNA-bound NR to the chromatin and/or transcription machinery (Voegel *et al.* 1998, Ma *et al.* 1999). The function of AD1 results from its ability to bind CBP and/or p300 and transmission of its activating signal by the acetylation activity of CBP and/or p300 on the chromatin and/or transcription machinery. Hence, CBP or p300 serves as a secondary coactivator of NRs because CBP or p300 can be recruited to the promoter by its interaction with the primary p160 coactivators. AD2 functions by an unknown mechanism that is independent of CBP and p300 (Yao *et al.* 1996, Voegel *et al.* 1998). Recently, coactivator-associated arginine methyltransferase 1 (CARM1), an intrinsic arginine methyltransferase, was found to play an important role in AD2 activation by direct interaction with AD2, implying the arginine methylation modification on the chromatin and/or transcription machinery (Chen *et al.* 1999). Other protein methyltransferases similar to the GRIP1-dependent coactivator of CARM1 may serve as secondary NR coactivators and synergistically enhance NR functions with the coexpression of CARM1 according to their histone or substrate specificities (Koh *et al.* 2001). Furthermore, the cross-talk of AD1 and AD2 activations may occur through the functional interaction between CARM1 and p300 in NR functions (Chen *et al.* 2000). A zinc-finger protein that regulates apoptosis and cell cycle arrest (Zac1), one of the proteins interacting with AD2 of the p160 coactivator, could serve as a primary coactivator or repressor of NR activity, and its

functions depend on the type of NR, promoter context and cell context (Spengler *et al.* 1997, Huang & Stallcup 2000).

In this study, which is a continuation of our previous screening work (Chen *et al.* 1999, Huang & Stallcup 2000), we screened a mouse 17-day embryo cDNA library, using the yeast two-hybrid system to search for additional components of the p160 coactivator complex that bind to the AD2 domain of GRIP1. This resulted in the identification of a mouse alpha actinin 2 protein, mACTN2. mACTN2 might work as a primary coactivator for NRs through the recruitment by NRs. mACTN2 also acts synergistically with GRIP1 to enhance NR functions through its interaction with and enhancement of the GRIP1 transactivation activity. Furthermore, the conserved LXXLL motif in mACTN2 and other related cytoskeletal proteins may have implications in the understanding of the cross-talk between cytoskeletal organization and NR functions through their roles in NR coactivator networks.

Materials and methods

Isolation of the mACTN2 cDNA clone

A partial mACTN2 cDNA clone (1.8 kb) was isolated by using the yeast two-hybrid system, as described previously (Chen *et al.* 1999), to screen a mouse 17-day embryo cDNA library for clones encoding proteins that bind to the C-terminal region (amino acids 1121–1462) of GRIP1. The full-length coding region of mACTN2 was synthesized by PCR, using the same mouse embryo library as template, a 5' sense primer 5'-TTGAATTCATGAATCAGATAGAGCCCGGC-3' (underlined is translation start codon) representing the 5' end of the mACTN2 coding sequence from our identified mRNA sequences based on hACTN2 (M86406), and an expressed sequence tag (EST) (accession no. AI119198), and a 3' antisense primer 5'-TTCTCGAGTTAGAGGT CGCTTTCCCGTAG-3' (underlined is translation stop codon) representing the 3' end of the coding region from the partial mACTN2 clone isolated from our yeast two-hybrid screen. All cDNA clones were sequenced by the dideoxy chain termination method, using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical, OH, USA), or by ABI automatic sequencer. The

mACTN2 sequence is deposited in GenBank file AY036877.

The tissue distribution of mACTN2 mRNA

A pair of gene-specific mACTN2 PCR primers (a 5' sense primer 5'-TTGAATTCATGAATCAGATAGAGCCCGGC-3' and a 3' antisense primer 5'-TTCTCGAGCTCATTCTCTTGATTCACAGC-3' encoding a truncated mACTN2 from amino acids 1–280, 840 bp) was used for a PCR amplification to analyze the mACTN2 tissue expression pattern in a mouse multiple-tissue cDNA (MTC) panel (no. K1423–1, Clontech). The PCR analysis was performed according to the manufacturer's protocol (PT3158–1, Clontech, CA, USA). The PCR cycling parameters for mACTN2 and G3 PDH mRNAs were 94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s cycled 30 times in a Perkin-Elmer 480 thermal cycler. All PCR experiments were repeated at least two times.

Plasmids

The complete mACTN2 coding region (codons 1–894) and other mACTN2 constructs (codons 1–280, 281–700 and 701–894) were synthesized by PCR and subcloned into the EcoRI and XhoI sites of vector pSG5.HA, which has promoters for expression *in vitro* and in mammalian cells, and provides an N-terminal HA-tag for the expressed protein (Chen *et al.* 1999). The pSG5.HA vectors coding for full-length GRIP1 (codons 5–1462) and GRIP1 (codons 5–1121) were described previously (Huang & Stallcup 2000); for GRIP1 (codons 563–1121) was cloned into an EcoRI-SalI fragment into the EcoRI and XhoI sites of vector pSG5.HA. Vectors encoding Gal4 DBD and VP16 AD fused to various fragments of mACTN2 were constructed by inserting EcoRI-XhoI fragments of the appropriate PCR-amplified mACTN2 cDNA into the EcoRI and SalI sites of the pM and pVP16 vectors (Clontech). Vectors encoding Gal4 DBD fused to various fragments of GRIP1s were constructed by inserting EcoRI-SalI fragments of the appropriate PCR-amplified GRIP1 cDNA into the EcoRI and SalI sites of the pM vector. Reporter genes, mouse mammary tumor virus promoter containing luciferase coding region (MMTV-LUC), MMTV(ERE)-LUC, EREII-LUC, MMTV(TRE)-LUC, and GK1 containing Gal4 response

elements, were described previously (Umesono *et al.* 1989, Paech *et al.* 1997). For expression of NRs in mammalian cells, vectors pSVAR₀ (Brinkmann *et al.* 1989) and pCMV.AR₀ (Chamberlain *et al.* 1994) for human AR, pHE0 (Green *et al.* 1988) for human ER α , and pCMX.hTR β 1 (Feng *et al.* 1998) for human TR β 1 were used as described previously.

Mutation in the LXXLL motif of mACTN2, mACTN2 LXXAA, was introduced into the pSG5.HA.mACTN2 vectors by the method of oligonucleotide-directed mutagenesis using uracil-containing single-strand DNA (Kunkel *et al.* 1987) and was verified by sequencing.

Cell culture and transient transfection assays

For functional assays, HeLa or HeLa S3 cells were grown in DMEM/F-12 supplemented with 10% charcoal/dextran-treated fetal bovine serum; C2C12 cells (ATCC) were grown in supplemented DMEM medium containing 10% fetal bovine serum. Transient transfections and luciferase assays were performed in a 24-well culture dish, as described previously (Ma *et al.* 1999). Total DNA was adjusted to 1 μ g by adding the necessary amount of pSG5.HA vector. Luciferase activity of the transfected cell extracts is presented as relative light units (RLU) and expressed as the mean and standard deviation from three transfected cultures (Ma *et al.* 1999). Since the expression of many control vectors that is used to monitor transfection efficiency is influenced by coactivators, internal controls were not used. Instead, reproducibility of observed effects was determined in multiple (at least three) independent transfection experiments.

Immunoprecipitation and immunoblots

For the association of mACTN2 with GRIP1 or AR, COS7 cells (Gluzman 1981) were transfected with these expression vectors. After transfection, cells were lysed in RIPA buffer (100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS and 1% Triton 100) at 4 °C. Lysates were subjected to immunoprecipitation with antibodies against either Gal4 DBD or AR (RK5C1 or N-20; Santa Cruz Biotechnology, CA, USA) for 3 h, followed by adsorption to Sepharose-coupled protein A/G (Santa Cruz Biotechnology) for 3 h. Immunoprecipitates were separated by SDS-PAGE and

analyzed by immunoblots. For determination of total protein levels, aliquots of cell lysates were subjected to direct immunoblots. Immunoblots were performed as previously described by using 5% of the extract from lysates for immunoprecipitation, and using antibodies against HA (3F10, Roche, Switzerland), Gal4 DBD or AR (N-20, Santa Cruz Biotechnology). All experiments were repeated at least three times.

Results

Isolation, sequence and tissue distribution of mACTN2

The C-terminal region of GRIP1 (amino acids 1121–1462) containing AD2 was used as the bait for screening a mouse 17-day embryo cDNA library in a yeast two-hybrid screening assay. One clone with 1693 unique nucleotides was found to be 90% identical to the sequence reported in GenBank (accession no. M86406) encoding an alpha actinin 2 from human skeletal muscle (hACTN2) (Beggs *et al.* 1992). Because our isolated clone lacked a complete 5' coding region, we used PCR to isolate the missing coding sequences from the same mouse 17-day embryo cDNA library; the upstream primers were designed from the 5' end of the previously reported hACTN2 and an ETS (GenBank accession no. AI119198), and the downstream primers represented the 3' end of the coding region of our newly isolated clone. Combined with these two newly synthesized PCR fragments and previously isolated clone, a 2936 bp cDNA clone encoding an 894 codon open reading frame (data not shown, deposited in GenBank accession no. AY036877) was identified to be 99% identical at the amino acid level to hACTN2. Our overlapped clones for mACTN2, containing a 179 bp fragment and a 75 bp fragment, were in the 5' and 3' non-translated regions respectively. Compared with the structural characteristics of hACTN2, the analysis of mACTN2 also showed two copies of the calponin homology domain at the N-terminus (amino acids 38–142 and 151–254), which are responsible for binding to alpha actin through the type I (⁴⁰QRKTFTAWCN⁴⁹) and type II (¹¹⁴LVSIGAEIIVDGNVKMTLGMIIWTII¹³⁸) actinin-type actin-binding motifs in the ACTN family. These calponin homology domains were followed by four copies of the spectrin repeat

Figure 1 Domains of mACTN2 and its tissue distribution. (A) Sequence motifs in mouse ACTN2 are analyzed and indicated by the ProfileScan Server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>). (B) The tissue expression pattern of mACTN2 mRNA and a positive control, G3 PDH mRNA, were performed in a mouse MTC panel (no. K1423–1, Clontech). Two mRNA expression pattern panels were run, and the PCR products analyzed by electrophoresis in a 1% agarose gel were expected to be an 840 bp fragment for mACTN2 and a 983 bp fragment for G3 PDH. Lanes 1–12 contained the indicated tissue. Lane 13 was a vector control containing mACTN2_{1–280} as a positive control, and lane M was a DNA ladder. We observed a similar expression pattern in two independent experiments.

(amino acids 277–331, 368–470, 479–588 and 604–682) and one Ca²⁺-binding EF hand motif (amino acids 749–818) (Fig. 1A).

We also analyzed the tissue expression pattern of mACTN2 mRNA in a mouse multiple tissue cDNA panel (Clontech, MTC, no. K1423–1) by PCR analysis. As shown in Fig. 1B, the highest mACTN2 mRNA expression was found in heart, lung and skeletal muscle (lanes 1, 4 and 6), whereas mACTN2 mRNA was expressed to a lesser extent in brain and testis (lanes 2 and 8). mACTN2 gene expression was undetectable in spleen, liver and kidney (lanes 3, 5 and 7). Interestingly, mACTN2 mRNA expression in the embryo gradually increased from day 7 to day 17 (lanes 9–12); however, no expression was found in the 7-day

embryonic stage (lane 9). Under the same PCR conditions, a control mRNA, G3PDH, expressed similar mRNA levels in all tissues and embryonic stages (Fig. 1B, bottom panel). Thus, the specificity of mACTN2 in tissues and embryonic stages might be an important factor for mouse development. However, the relationship of the expression in specific tissues and embryonic stages to the functions of mACTN2 remains to be investigated.

Binding of mACTN2 to GRIP1 enhances GRIP1 transactivation activities

Our previous yeast two-hybrid screening results showed that mACTN2 binds to the C-terminal region of GRIP1 (amino acids 1122–1462; data not

shown). We performed co-immunoprecipitation experiments to confirm this protein–protein interaction in COS7 cells and further dissected the binding domains of mACTN2 in GRIP1. We used an expression vector containing full-length mACTN2 fused to the yeast Gal4 DNA-binding domain (Gal4 DBD) for pulling down three hemagglutinin A (HA)-tagged GRIP1 fragments (amino acids 5–1121, 563–1462 and 563–1121) with antibody against Gal4 DBD in COS7 cell lysates. The pull-down result showed that mACTN2 bound only to a GRIP1 fragment containing the C-terminal region of GRIP1 (amino acids 1122–1462), such as GRIP1_{563–1462}, but did not bind to GRIP1_{5–1121} or GRIP1_{563–1121} in COS7 cells (Fig. 2A, upper panel, lanes 1–3). A similar conclusion was reached in two independent

co-immunoprecipitation experiments in COS7 cells (data not shown). We failed to determine the direct binding domains between mACTN2 and GRIP1 using the glutathione *S*-transferase pull-down assay (data not shown). Hence, these findings suggest that mACTN2 might indirectly interact with GRIP1 under *in vivo* conditions (such as in COS7 cells) and the GRIP1 C-terminal region might be an important region for binding.

Because mACTN2 interacted *in vivo* with GRIP1 (Fig. 2A), we tested whether mACTN2 could affect the transactivation activity (AD1 and/or AD2) of GRIP1 through various GRIP1 fragments, containing AD1, AD2 or both, fused to the yeast Gal4 DBD, which is capable of activating transcription of a reporter gene containing Gal4 response elements. mACTN2 was able to enhance both the AD1 and AD2 transactivation activities of GRIP1 (Fig. 2B; compare histograms 1, 3, 5 and 6). One possible repression domain (amino acids 1122–1304) in GRIP1 played a steric hindrance role for the regulation of its transactivation activity because full-length GRIP1 and GRIP1_{1122–1462} have lower activities than fragments that lack this repression region, such as GRIP1_{5–1121} and GRIP1_{1305–1462} (Huang & Hsu, unpublished data). Alternatively, mACTN2 might not be able to compete for this repression region and then fail to enhance transactivation activities in full-length GRIP1 or GRIP1_{1122–1462} (Fig. 2B; compare histograms 1, 2 and 4). In addition, mACTN2 might indirectly

Figure 2 Physical and functional interactions between mACTN2 and GRIP1. (A) COS7 cells were transfected with HA.GRIP1_{5–1121}, HA.GRIP1_{563–1462} or HA.GRIP1_{563–1121} (5 µg) in the absence or presence of Gal4 DBD.mACTN2 (5 µg). Cell extracts were subjected to immunoprecipitation with anti-Gal4 DBD antibody and then immunoblotted with anti-HA antibody (upper panel) or immunoblotted with anti-HA or anti-Gal4 DBD antibody for 5% cell extracts (input controls, middle and lower panels) applied in immunoprecipitation. We observed a similar binding pattern in two independent experiments. (B) HeLa S3 cells were treated with 0.4 µg mACTN2, 0.3 µg Gal4 DBD or various GRIP1 fragments (amino acids 5–1462, 5–1121, 1122–1462, 1305–1462 and 563–1121) fused with Gal4 DBD, and 0.3 µg of the GK1 reporter gene, which encodes luciferase and is controlled by Gal4 response elements. Fold activation (indicated above the bars) was calculated by comparing the luciferase activity of each GRIP1 fragment fused with Gal4 DBD cotransfected with mACTN2 to those not cotransfected with mACTN2. The data (B) are based on at least three separate experiments.

regulate the GRIP1 transactivation activity because mACTN2 failed to interact directly with GRIP1_{5–1121} or GRIP1_{563–1121} (Fig. 2A).

The enhancement of mACTN2 homodimerization by GRIP1

Figure 2 shows that mACTN2 could enhance GRIP1 transactivation activities through their physical interaction or by unknown mechanisms. We investigated the possible effect of GRIP1 on mACTN2 characteristics, such as homodimerization. In the mammalian two-hybrid system, the transient transfection assays in HeLa cells demonstrated that full-length or other truncated mACTN2 fused to yeast Gal4 DBD (pM) vectors had no transactivation activity when compared with the value of the pM vector along with the vector containing VP16 activation domain (pVP16) through monitoring the luciferase activity change in the Gal4 responsive element reporter (compare Fig. 3A, closed columns). Subsequently, the full-length mACTN2 was inserted into the pVP16 vector to confirm its homodimerization property and define the major binding region in mACTN2 (compare Fig. 3A, open columns). The full-length mACTN2 demonstrated the strongest protein–protein interactions (by 463-fold) in all tested ACTN2 fragments (Fig. 3A, histogram 1, open column). However, the region of spectrin repeats (amino acids 281–700, by 13-fold) of mACTN2 was the only truncated fragment involved in mACTN2 dimerization in cells (Fig. 3A; compare histograms 2–4, open columns). Hence, our findings suggest that its proper conformation and the spectrin repeats of mACTN2 are two major factors for its dimerization in cells. We also found a stronger homodimerization property in the full-length mACTN2 when a higher transfected dose was applied in the mammalian two-hybrid assays (data not shown). Importantly, the combination of pM.ACTN2 and pVP16.ACTN2 was specifically enhanced by the presence of either full-length GRIP1 or C-truncated GRIP1 (GRIP1_{5–1121}) in the mammalian two-hybrid assays (compare Figs 3B and C). In the presence of the highest dose of GRIP1_{5–1462} and GRIP1_{5–1121}, these interactions were enhanced fourfold and 32-fold respectively (Fig. 3B). However, a similar dose of GRIP1_{5–1462} and GRIP1_{5–1121} showed no enhancement com-

pared with the combination of pM and pVP16.ACTN2 (Fig. 3C; compare histograms 2–4) or pM.ACTN2 and pVP16 (Fig. 3C; compare histograms 5–7).

The conserved NR binding motif, LXXLL, of mACTN2 and its family proteins

Of all reported structural domains in the alpha actinin family, we identified one NR binding motif, LXXLL, near to the mACTN2 N-terminus (amino acids 72–76). The LXXLL motif is conserved in all alpha-actinin family proteins, including those from mouse, human, chicken and *Drosophila* (Fig. 4A). Interestingly, one or more LXXLL motifs could be found in other related family proteins, such as β -spectrin, plectin and kakapo (Fig. 4A and data not shown). In addition to the conserved LXXLL motif, other highly conserved sequences, covering all reported functional domains in the ACTN family (Fig. 1A), suggest that ACTN family members may have largely redundant functions in the cell. As the LXXLL motif is a signature for NR coactivators (Feng *et al.* 1998, McInerney *et al.* 1998), we tested whether mACTN2 could interact with AR through this motif by the co-immunoprecipitation assay in COS7 cells. Our data indicated that wild-type mACTN2, containing an LXXLL motif and a mutation of the LXXLL motif in mACTN2 to LXXAA (A stands for alanine) both bound to AR, but the mutant form had a much weaker ability than wild-type mACTN2 (compare Fig. 4B, lanes 2 and 3 respectively). Hence, our findings suggest that the LXXLL motif might be one of the functional domains for mACTN2 or other ACTN proteins that interact with AR or other NRs.

mACTN2 is a primary coactivator for NRs and acts synergistically with GRIP1 in NR functions in HeLa cells

Because mACTN2 could complex with GRIP1 and AR (Figs 2A and 4B), we examined the possibility that mACTN2 could serve as a primary nuclear receptor coactivator. In transient transfection assays, we found that mACTN2 not only served as a primary NR coactivator in AR, ER and TR functions by seven- to eightfold, but also

Figure 3 Enhancement of the mACTN2 homodimerization by GRIP1. (A) HeLa S3 cells were transiently transfected with 0.4 μ g pM or various pM.mACTN2 fragments paired with 0.4 μ g pVP16 (closed columns) or PVP16.mACTN2 (open columns), along with the GK1 reporter gene (0.2 μ g). Luciferase activities of the transfected cell extracts were determined. Numbers above the bars indicate fold activation compared with that of the pM and pVP16. (B) HeLa S3 cells were transiently transfected with 0.2 μ g pM.mACTN2 and 0.2 μ g VP16.mACTN2 in the presence of the indicated amount of either GRIP1₅₋₁₄₆₂ (solid circles) or GRIP1₅₋₁₁₂₁ (open circles), along with the GK1 reporter gene (0.2 μ g). (C) HeLa S3 cells were transiently transfected with 0.2 μ g pM or pM.mACTN2 paired with 0.2 μ g pVP16 or pVP16.mACTN2 in the absence or presence of 0.4 μ g GRIP1₅₋₁₄₆₂ or GRIP1₅₋₁₁₂₁, along with the GK1 reporter gene (0.2 μ g). RLU: relative light units. These data (A, B and C) are the average of three experiments (mean $\pm$ S.D.; $n=3$).

synergistically interacted with GRIP1 in these three NR functions to 2.2-, 6.5- and 18.1-fold respectively (Fig. 5). The synergy ratio was calculated by dividing the extra activity observed when mACTN2 and GRIP1 were added together, by the sum of the extra activity due to mACTN2 alone, plus the extra activity due to GRIP1 alone.

Our results showed that mACTN2 could synergistically enhance GRIP1-induced NR coactivator functions in the presence of cognate ligands (Fig. 5). We further examined whether the synergy effect of mACTN2 on GRIP1-induced NR coactivator functions depended on each cognate ligand. In transiently transfected assays, we found

A

Gene Product	Homology, %	NR box	Access number
ACTN2 [Mus musculus].	100	67 R NGLKLM L L L E V I S G	AAF76325
ACTN2 [Homo sapiens].	99	67 R NGLKLM L L L E V I S G	NP_001094
ACTN1 [Rattus norvegicus].	82	60 R DGLKLM L L L E V I S G	AAD12064
ACTN1 [Homo sapiens].	82	60 R DGLKLM L L L E V I S G	NP_001093
ACTN3 [Mus musculus].	83	73 R NGLKLM L L L E V I S G	NP_038484
ACTN3 [Homo sapiens].	82	74 R NGLKLM L L L E V I S G	NP_001095
ACTN4 [Mus musculus].	79	80 R DGLKLM L L L E V I S G	CAC10069
ACTN4 [Homo sapiens].	80	52 R DGLKLM L L L E V I S G	NP_004915
ACTN [Chicken].	96	70 R NGLKLM L L L E V I S G	F20111
ACTN [Drosophila melanogaster].	70	61 R NGLKLM L L L E V I S G	AAF45705
beta-spectrin [Homo sapiens].	41	83 R DGRMLIK L L L E V L S G	NP_000338
beta-spectrin 1 [Mus sp.].	41	83 R DGRMLIK L L L E V L S G	NP_038703
beta-spectrin 1 [Homo sapiens].	43	83 R DGRMLIK L L L E V L S G	NP_003119
beta-spectrin 2 [Mus musculus].	42	70 R DGRMLIK L L L E V L S G	NP_033286
beta-spectrin 2 [Homo sapiens].	41	86 R DGRNLLR L L L E V L S G	NP_008877
beta-spectrin 3 [Rattus norvegicus].	43	86 R DGRNLLR L L L E V L S G	NP_062040
beta spectrin [fruit fly].	31	79 R DGRMLIK L L L E V L S G	226515
plec 1,24pha [Mus musculus].	19	219 R DGHNLIS L L L E V L S G	AF188008_1
plectin [Homo sapiens].	23	208 R DGHNLIS L L L E V L S G	CAA91196
Kakapo [Drosophila melanogaster].	24	178 L R DGNLLS L L L E V L S G	CAA09869
LXXLL			

B

Figure 4 A conserved LXXLL motif in mACTN2 and other family proteins. (A) Analysis of the LXXLL motif from all alpha actinin family proteins, the beta spectrin family and plectin was identical in the consensus sequence. The homology value is the percent similarity in amino acid sequence between full-length mACTN2 and proteins of the actin-binding family from various species. (B) COS7 cells were transfected with HA.ACTN2_{1–894} (LXXLL), HA.ACTN2_{1–894} (LXXAA) or HA (5 µg) in the presence of androgen receptor (pCMV.AR₀, 5 µg). Cell extracts were subjected to immunoprecipitation with anti-AR antibody and then immunoblotted with anti-HA antibody (upper panel) or immunoblotted with anti-AR or anti-HA antibody for 5% of cell extracts (input controls, middle and lower panels) by immunoprecipitation. We observed a similar binding pattern in two independent experiments.

that mACTN2 could still serve as a primary NR coactivator for AR function (1·7-fold), ER function (sevenfold) and TR function (1·6-fold) (Fig. 6; compare histogram 1, closed and open columns) in HeLa cells, grown in the absence of their appropriate hormones. GRIP1 showed 2·7-, 20- and twofold enhancements in AR, ER and TR functions respectively (Fig. 6; compare histograms 1 and 2, closed columns). mACTN2 could further synergistically enhance GRIP1 coactivator functions in AR, ER and TR functions to 2·5-, 1·4- and 5·3-fold respectively (Fig. 6; compare histograms 1 and 2). In summary, mACTN2 could synergistically enhance the coactivation of GRIP1 in the absence and presence of the appropriate NR hormones (Figs 5 and 6).

The LXXLL motif is not the only functional domain for mACTN2 coactivation in NR systems

Data in Fig. 5 show that mACTN2 served as a primary NR coactivator as well as a secondary NR coactivator (GRIP1-dependent manner). We used three mACTN2 truncated fragments (amino acids 1–280, 281–700 and 701–894) in HeLa cells to compare their primary and secondary coactivator functions with full-length mACTN2 in these NR functions. The fragment of mACTN2 containing the LXXLL motif (amino acids 1–280) kept most of the primary coactivator function in the AR system, but not in ER and TR (Table 1). The other two fragments expressed at 25–30% of the level of

full-length mACTN2 primary coactivator activity in the AR system, but had a similar effect (around 50% level of full-length mACTN2) to the primary coactivator activity and fragment 1–280 in the ER and TR systems. The synergistic effect (compared with full-length mACTN2) of these three mACTN2 fragments in the GRIP1 coactivator system had similar synergy ratios in the AR and TR systems, but had 50% of the synergy ratio in the ER system. Considered together, the data in Table 1 suggest that the LXXLL motif in mACTN2 might not be the only functional motif for the primary and secondary coactivator functions in mACTN2 for NR functions.

The secondary coactivator functions of mACTN2 is synergistic and dependent on GRIP1 in C2C12 cells

To clarify the NR coactivator role of mACTN2 in muscle and heart development, we used the C2C12 myoblast cell line, derived from mouse satellite cells, as a model because the cell normally expresses ACTN2 (Hance *et al.* 1999). In transient transfection assays, we found that mACTN2 (wild-type or LXXAA mutant) synergistically interacted with GRIP1 in AR, ER and TR functions to 10 (or 12)-, 2.5 (or 2.3)- and 22 (or 17)-fold respectively (Figs 7A–C, histograms 5 and 6). mACTN2, but not GRIP1, lost the ability to serve the role of the primary NR coactivator in C2C12 cells (Figs 7A–C; compare histograms 1–4). Similar to the finding in HeLa cells, the LXXLL motif is not the only function motif of mACTN2 in C2C12 cells (Figs 7A–C; compare histograms 5 and 6). In the absence of appropriate NR hormones, mACTN2 and GRIP1 both in combination synergistically enhanced AR and ER functions in C2C12 cells

Figure 5 Functional interactions between mACTN2 and GRIP1 on nuclear receptor functions. (A) HeLa cells were transiently transfected with MMTV-LUC reporter gene (0.3 µg), pSVAR₀ (0.2 µg) encoding AR, and 0.25 µg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 µg). Transfected cultures were grown in 100 nM dihydrotestosterone (DHT), and luciferase activities of the transfected cell extracts were determined. Numbers above the columns indicate fold activity relative to that of hormone-activated AR with no added coactivators. (B) HeLa cells were transiently transfected with MMTV(ERE)-LUC reporter gene (0.3 µg), pHE0 (0.01 µg) encoding hERα, and 0.25 µg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 µg). Transfected cultures were grown in 100 nM estradiol (E2) and luciferase activities of the transfected cell extracts were determined. Numbers above the columns indicate fold activity relative to that of hormone-activated ER with no added coactivators. (C) HeLa cells were transiently transfected with MMTV(TRE)-LUC reporter gene (0.3 µg), pCMX-hTRβ1 (0.01 µg) encoding hTRβ1, and 0.25 µg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 µg). Transfected cultures were grown in 100 nM 3,5,5'-triiodo-L-thyronine (T₃) and luciferase activities of the transfected cell extracts were determined. Numbers above the columns indicate fold activity relative to that of hormone-activated TR with no added coactivators. RLU: relative light units. These data (A, B and C) are the average of four experiments (mean±S.D.; n=4).

(data not shown). All synergy effects by mACTN2 and GRIP1 could not be reached by simply increasing mACTN2 or GRIP1 amount alone to the sum of mACTN2 and GRIP1 (data not shown).

The interesting issue is whether the effect of mACTN2 on non-receptor-dependent promoters exists because of the multiple coactivator functions of some nuclear receptor coactivators. C2C12 and HeLa cells were transfected with a c-Fos promoter (Fos-LUC) and three copies of the cAMP response element [$3 \times (\text{CRE})\text{-LUC}$] in the presence of GRIP1 or ACTN2 (wild-type or LXXAA mutant), or both in combination (Figs 7D and E). GRIP1 or mACTN2 (wild-type or LXXAA mutant) alone had little or no effect on these tested reporters in these two cells (Figs 7D and E; compare histograms

1–4). The enhancement effects on Fos-LUC and $3 \times (\text{CRE})\text{-LUC}$ reporters caused by mACTN2 (wild-type or LXXAA mutant) and GRIP1 together in C2C12 cells were synergistic (Figs 7D and E; compare histograms 5 and 6, closed columns), whereas the enhancement effects in HeLa cells on Fos-LUC reporter were synergistic and $3 \times (\text{CRE})\text{-LUC}$ reporter was additive (Figs 7D and E; compare histograms 5 and 6, open columns). Here, our data suggest that mACTN2 and GRIP1 both in combination might serve as a general coactivator for some receptor non-receptor-dependent transcriptional systems.

Discussion

The coactivator role of mACTN2 in NR functions

In this work, we found that mACTN2 could interact with GRIP1 in the yeast two-hybrid

Figure 6 The synergy effect by mACTN2 in GRIP1's NR coactivator functions is independent of cognate ligands. (A) HeLa cells were transiently transfected with MMTV-LUC reporter gene (0.3 μg), pSVAR₀ (0.2 μg) encoding AR, and 0.25 μg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 μg). Transfected cultures were grown in DMEM supplemented with 10% charcoal-treated fetal bovine serum, and luciferase activities of the transfected cell extracts were determined. Numbers above the column indicate fold activity relative to that of AR with no added coactivators. (B) HeLa cells were transiently transfected with EREII-LUC reporter gene (0.3 μg), pHE0 (0.01 μg) encoding hER α , and 0.25 μg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 μg). Transfected cultures were grown in DMEM supplemented with 10% charcoal-treated fetal bovine serum, and luciferase activities of the transfected cell extracts were determined. Numbers above the column indicate fold activity relative to that of ER with no added coactivators. (C) HeLa cells were transiently transfected with MMTV(TRE)-LUC reporter gene (0.3 μg), pCMX-hTR β 1 (0.01 μg) encoding hTR β 1, and 0.25 μg pSG5.HA-GRIP1 in the absence (closed columns) or presence (open columns) of mACTN2 (0.25 μg). Transfected cultures were grown in DMEM supplemented with 10% charcoal-treated fetal bovine serum, and luciferase activities of the transfected cell extracts were determined. Numbers above the columns indicate fold activity relative to that of TR with no added coactivators. RLU: relative light units. These data (A, B and C) are the average of three experiments (mean $\pm$ S.D.; $n=3$).

Table 1 The LXXLL motif of mACTN2, amino acids 72–76, is not the only functional motif for its primary or secondary coactivator functions

Protein(s)	Fold activation					
	AR+MMTV-LUC		ER+MMTV(ERE)-LUC		TR+MMTV(TRE)-LUC	
	–GRIP1	+GRIP1	–GRIP1	+GRIP1	–GRIP1	+GRIP1
Vector ^a	1±0.2	7.6±0.6	1±0.1	30±0.3	1±0.1	13±0.6
mACTN2 _{1–894}	8.9±0.8	33±0.6	8.2±0.7	237±22	6.9±0.1	325±3
mACTN2 _{1–280}	6.9±0.6	31±4	4.6±0.5	105±9	3.1±0.6	218±5
mACTN2 _{281–700}	2.1±0.2	17±2	2.6±0.2	134±24	2.2±0.5	201±28
mACTN2 _{701–894}	2.8±0.8	12±0.4	3.5±0.1	103±16	3.6±0.2	196±9

^aHeLa cells were transfected with plasmids described in Fig. 5. Other mACTN2 fragments were performed in the same conditions of full-length mACTN2 in HeLa cells. Similar activity profiles were observed in four independent experiments.

screening and co-immunoprecipitation assays (Fig. 2A and data not shown). We showed that the LXXLL motif, a signature for NR coactivators, is conserved in mACTN2 and other alpha actinin family proteins, including different subfamilies and species (Fig. 4A). One of the findings in this study was the identification of a unique LXXLL motif in mACTN2 that played a major role in the interaction with AR in COS7 cells (Fig. 4B). However, the LXXLL motif of mACTN2 was shown to be a dispensable motif for its primary coactivator role in NR functions, as two truncated mACTN2s (encoding 281–700 and 701–894) lacking the LXXLL motif retained 50% or more of its coactivator function compared with full-length mACTN2 NR transcriptional activations (Table 1). Furthermore, the mutant mACTN2 (LXXAA) of full-length or amino acids 1–280 constructs retained the primary and secondary coactivator functions of wild-type mACTN2 (data not shown). These findings suggest that the primary NR coactivator of mACTN2 in NR transcription functions might act through an alternative, and as yet unknown, mechanism. At least, this conclusion is supported by our previous study, which found that the sole LXXLL motif is not an essential motif for primary NR coactivation of another GRIP1 interaction protein, Zac1 (Huang & Stallcup 2000). Therefore, an mACTN2 fragment lacking the LXXLL motif still could act as a primary coactivator through a similar mechanism to Zac1 or a novel pathway. However, it remains to be investigated how mACTN2 can serve as a primary NR coactivator without a transcriptional activation domain (Fig. 3A, closed columns). In addition, the property of functional NR coactivation preference

by mACTN2 could be explained by a study of the NR-binding preferences of multiple LXXLL motifs in p160 coactivators. GRIP1, a p160 coactivator, contains at least three NR binding motifs, NR box or LXXLL. NR box II of GRIP1 prefers to interact with ER, and NR box III strongly interacts with GR and AR (Ding *et al.* 1998, McInerney *et al.* 1998). Thus, the NR selectivity by NR box in GRIP1 suggests the LXXLL motif and other unidentified domains in mACTN2 may be equally important for its physical and functional interactions in various NR systems.

The synergism of mACTN2 and GRIP1 on NR functions

In addition to revealing that mACTN2 could interact with GRIP1 and AR (Figs 2A and 4B), our studies also demonstrated that mACTN2 and GRIP1 acted synergistically in NR functions (Figs 5–7). mACTN2 not only expressed similar primary coactivator functions on AR, ER and TR, but also had synergistic effects with GRIP1 on these NR functions (Fig. 5). These synergy effects might be through the enhancement of GRIP1 transcriptional activities through the physical interaction between mACTN2 and GRIP1 (Fig. 2). We also detected a similar synergy effect with GRIP1 in mACTN2 (LXXAA) mutants (Fig. 7 and data not shown). Our data suggest that the full coactivation of mACTN2 might be through its LXXLL motif, GRIP1 interacting domain(s) and other unidentified functional domain(s) (Table 1), or be replaced by the redundancy of ACTN proteins in cells (Fig. 4A). Alternatively, the complexity

Figure 7 Functional interactions between mACTN2 and GRIP1 on receptor- and non-receptor-dependent promoters in C2C12 cells. (A) C2C12 cells were transiently transfected with MMTV-LUC reporter gene (0.25 µg), pSVAR₀ (0.1 µg) encoding AR, and 0.325 µg pSG5.HA-GRIP1, mACTN2 (wild-type or LXXAA mutant) or both in combination. Transfected cultures were grown in 10 nM dihydrotestosterone (DHT), and luciferase activities of the transfected cell extracts were determined. Relative fold indicates fold activity relative to that of hormone-activated AR with no added coactivators. (B) C2C12 cells were transiently transfected with MMTV(ERE)-LUC reporter gene (0.25 µg), pHE0 (0.1 µg) encoding hER α , and 0.325 µg pSG5.HA-GRIP1, mACTN2 (wild-type or LXXAA mutant) or both in combination. Transfected cultures were grown in 10 nM estradiol (E2) and luciferase activities of the transfected cell extracts were determined. Relative fold indicates fold activity relative to that of hormone-activated ER with no added coactivators. (C) C2C12 cells were transiently transfected with MMTV(TRE)-LUC reporter gene (0.25 µg), pCMX-hTR β 1 (0.1 µg) encoding hTR β 1, and 0.325 µg pSG5.HA-GRIP1, mACTN2 (wild-type or LXXAA mutant) or both in combination. Transfected cultures were grown in 10 nM 3,5,5'-triiodo-L-thyronine (T₃), and luciferase activities of the transfected cell extracts were determined. Relative fold indicates fold activity relative to that of hormone-activated TR with no added coactivators. (D and E) C2C12 (closed columns) and HeLa (open columns) cells were transiently transfected with Fos-LUC reporter gene (0.3 µg) or 3x(CRE)-LUC reporter gene (0.3 µg) and 0.325 µg pSG5.HA-GRIP1, mACTN2 (wild-type or LXXAA mutant) or both in combination. Transfected cultures were treated with 1 µg/ml for 20 h in Fos-LUC reporter gene. Luciferase activities of the transfected cell extracts were determined. Relative fold indicates fold activity relative to that with no added coactivators. These data are the average of three experiments (mean $\pm$ s.d.; $n=3$).

(or conformation) level of full-length mACTN2, *in vivo*, might be important for its functional roles in cells because of its homodimerization difference between full-length and a fragment of amino acids

281–700 (Fig. 3A). These data suggest that other unknown regulatory factors for mACTN2 in cells may be involved in the final functional complexes among mACTN2, GRIP1 and NRs.

Selectivity and redundancy of mACTN2

mACTN2 was not expressed in all tissues and embryo stages (Fig. 1B). The mACTN2 expression level in heart was equal to or greater than that in skeletal muscle. In addition, the regulation of muscle differentiation by some nuclear receptor coactivators implies that the coactivator role of mACTN2 and other NR coactivators might be important for the normal function or development of muscle and heart. A recent study maps the familial polymorphic ventricular arrhythmias in arrhythmogenic right ventricular cardiomyopathy to chromosome 1q42–43, in close proximity to the α -actinin 2 locus (Beggs *et al.* 1992, Bauce *et al.* 2000). Another study shows that p300, a coactivator of myocyte enhancer binding factor 2C (MEF2C), and *Drosophila* NK2 transcription factor related, locus 5, are two of three pivotal regulators of cardiac gene expression (Poizat *et al.* 2000). These two studies suggest that α -actinin 2 and its nuclear receptor coactivator functions might play important roles in heart development, because GRIP1 is also an important coactivator for the MEF2 family. The latest study shows that mutations in the muscle LIM protein and α -actinin 2 genes are found in dilated cardiomyopathy and endocardial fibroelastosis (Mohapatra *et al.* 2003). These mutations primarily disrupt the physical interaction between the muscle LIM protein and α -actinin 2 genes and alter the cellular localizations of these two genes in cells.

The mechanism involving mACTN2 in NR coactivator functions is still unclear. However, the subcellular localization (cytoplasm or nucleus) of mACTN2 might be a regulatory factor for its working mechanisms, because the family of α -actinin interacts with a broad spectrum of molecules (Burridge *et al.* 1990, Colombo *et al.* 1993). Recently, an actin-binding protein, filamin, a 280 kDa component of the cytoskeleton, was found to interact with hAR and facilitate hAR nuclear translocation (Ozanne *et al.* 2000). The study by Honda *et al.* (1998) indicated that ACTN4 can translocate into the nucleus, depending on the cell type, inhibition of phosphatidylinositol 3 kinase or actin depolymerization. Hence, the possibility of redundant functions from other ACTN proteins and that the unidentified regulatory intracellular translocation of mACTN2 will affect the final functional roles of mACTN2 in cells. Nonetheless,

a detailed mechanistic analysis of actin-binding proteins containing the LXXLL motif, involvement in cytoskeleton remodeling and transcriptional activation by NRs could increase our understanding of how actin-filament webs influence gene expression during both normal development and pathophysiological conditions.

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