The Co-repressor mSin3A Is a Functional Component of the REST-CoREST Repressor Complex*

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The repressor REST/NRSF restricts expression of a large set of genes to neurons by suppressing their expression in non-neural tissues. We find that REST repression involves two distinct repressor proteins. One of these, CoREST, interacts with the COOH-terminal repressor domain of REST (Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallmanning, J., Ballas, N., and Mandel, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9873–9878). Here we show that the co-repressor mSin3A also interacts with REST. The REST-mSin3A association involves the NH2-terminal repressor domain of REST and the paired amphipathic helix 2 domain of mSin3A. REST forms complexes with endogenous mSin3A in mammalian cells, and both mSin3A and CoREST interact with REST in intact mammalian cells. REST repression is blocked in yeast lacking Sin3 and rescued in its presence. In mammalian cells, repression by REST is reduced when binding to mSin3A is inhibited. In mouse embryos, the distribution of mSin3A and REST transcripts is largely coincident. The pattern of CoREST gene expression is more restricted, suggesting that mSin3A is required constitutively for REST repression, whereas CoREST is recruited for more specialized repressor functions.

Many genes essential for neuronal functioning, including the brain type II voltage-dependent sodium channel, neuronal growth factors, and neurotransmitter receptors, are repressed in non-neuronal cells by the transcriptional repressor, REST/NRSF (2–5). Removal of the REST/NRSF binding site (RE1/NRSE) from the regulatory region of transgenes (6) or expression of a dominant negative form of REST/NRSF in vivo (5) results in aberrant expression of the target genes in non-neural tissues. Deletion of the mouse REST/NRSF gene by homologous recombination results in embryonic lethality (5). Because of its importance in establishing and maintaining the expression pattern of a large number of genes required for neuronal functioning, it was of interest to identify the molecules involved in the repressor mechanism.

Previous studies identified two distinct domains in the NH2 and COOH termini of REST that were both necessary and sufficient to repress brain type II sodium channel reporter genes in transient transfection assays (7) and showed that repression by each of these two domains required distinct titratable factors (8). Recently, repression from the COOH-terminal domain was determined to be mediated by the co-repressor, CoREST (1). We sought to determine whether, in addition to CoREST, mSin3A, a co-repressor for several regulated repressor complexes, might also be involved in REST repression. We found that mSin3A is indeed a functional co-repressor for REST. mSin3A interacts with REST in vitro, in yeast and in intact mammalian cells, and interestingly, the binding site maps to the NH2-terminal repressor domain in REST. Furthermore, experiments both in yeast and mammalian cells showed that mSin3A is involved in repressor function. In vivo, the expression patterns of the co-repressors mSin3A and CoREST are distinct. Specifically, in early embryogenesis CoREST exhibits a much more restricted pattern of expression compared with REST and mSin3A, suggesting that the composition of the REST repressor complex during development is dynamic.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

Yeast Two-hybrid Constructs—LexA-mSin3A was obtained by cloning full-length mSin3A into PBTM116 by standard PCR1 cloning techniques. pGADmSin3A was made by cloning full-length mSin3A (provided by Carol Laherty and Bob Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA) into pGADGH (donated by Rolf Sternglanz, SUNY, Stony Brook, NY) using standard PCR-based cloning techniques. pGADNmSin3A was obtained by cloning a restriction fragment encoding the first 530 amino acids of mSin3A into pGADGH. To make a fusion between full-length CoREST and the LexA DNA binding domain, a 3030-base pair NcoI–PstI fragment from pCDNA1.1CoREST (1) was cloned between the Smal and PstI sites of the vector pBTM to generate pBTMCoREST. A fusion of the LexA DNA binding domain to amino acids 102–482 of REST has been described previously (1). A fusion of the LexA DNA binding domain to amino acids 1–293 of CoREST was made by cloning an EcoRI/EcoRI fragment of Gal4-CoREST (amino acids 1–293)2 into pBTM. Fusions between the LexA DNA binding domain and the amino or carboxyl portions of REST (N-REST and C-REST, respectively) have been described previously (1).

1 The abbreviations used are: PCR, polymerase chain reaction; PAH, paired amphipathic helix; RE1, repressor element 1; GST, glutathione S-transferase; NLS, nuclear localization signal; PBS, phosphate-buffered saline; pBST, PBS-Triton X-100; WT, wild type; N-REST and C-REST, NH2-terminal and COOH-terminal portions of REST, respectively.

N. Ballas, unpublished.
GST Fusion Constructs for Bacterial Expression—GST-REST (32–122) was made by subcloning an EcoRI BamHI fragment of VP16 REST (obtained in the two-hybrid screen) into pGEX-2X.

Mammalian Expression Constructs—An NH2-terminal Flag epitope was added to REST by cloning it into pCMV-Tagl (Stratagene) by standard PCR-based cloning techniques (RESTITANS FLAG, nucleotides 1535–1566 (encoding the consensus NLS) of REST-FLAG were removed and replaced with a BamHI site by PCR, and the RESTITANS Flag coding sequence was subcloned into pcDNA3.1+ (Invitrogen). The pTet-On plasmid was purchased from CLONTECH, and the tetracycline-inducible REST expression vector, pTRE-REST, was made by cloning REST-FLAG into pTRE (CLONTECH) at the EcoRI site. To generate REST Myc, the coding sequence of REST was cloned into pcDNA3.1+ (Invitrogen) by standard PCR-based cloning techniques. mSin3A Myc was generously donated by Carol Laherty and Bob Eisenman. CoREST Myc has been described previously (1). The REI-containing type II sodium channel reporter gene, pSdK7, has been described previously (9).

In Situ Hybridization Probes—A plasmid containing a 1.1-kilobase pair cDNA fragment spanning the zing finger DNA binding domain of mouse REST (mREST in pBluescript) has been described previously (2). Linearization of this plasmid with EcoRI and transcription with T7 RNA polymerase were used to generate antisense transcripts, while digestion with KpnI and transcription with T3 RNA polymerase were used to generate sense transcripts. A mouse expressed sequence tag contained within a 1.3-kilobase pair insert spanning the entire open reading frame of CoREST (identified by homology with human CoREST) was purchased from Genome Systems Inc. Linearization with BamHI and transcription with T7 RNA polymerase yielded the antisense transcript, while digestion with Xhol and transcription with T3 RNA polymerase yielded a sense transcript. An 800-base pair NsiI/HindIII fragment spanning amino acids 274–533 of mSin3A was cloned into pBluescript II KS. Antisense and sense riboprobes were transcribed from plasmids linearized with SpeI or EcoRI, using T7 and T3 RNA polymerases, respectively.

All fusion protein constructs were sequenced across the junction to ensure that inserts were in frame. All constructs generated by PCR were sequenced completely.

Yeast Two-Hybrid Assay

pBTM116-Sin3A was transformed into L40 yeast together with a mouse 9.5–10.5 days postcoitum cDNA library ligated into pVP16 as described (10). An estimated 5 × 10^11 transformants were screened. Proteins interacting with mSin3A were identified by growth on selective medium in the presence of 5 mM 3-aminotriazole and confirmed by assaying for β-galactosidase activity. Plasmids rescued from yeast were transformed into L40 together with either pBTM116-mSin3A or pBTM116-lamin to assess the specificity of the interaction. Positively interacting clones were characterized by sequence analysis. Direct yeast interaction assays were performed using yeast transformed with either LexA-REST, LexA-NREST, or LexA-CREST and either pGAD-mSin3A, pGAD-NmSin3A, or pGAD-CoREST. Activity of HIS3 and LacZ reporter genes was used to identify positive interactions.

GST Pull-down Assays

Expression and purification of GST-fusion proteins and GST pull-down assays were performed as described (11). In vitro transcription/translation was performed using the rabbit reticulocyte lysate-based TNT system from Promega according to the manufacturer’s instructions. The in vitro translatable mSin3A deletion series in pCS2+ MT was kindly provided by Carol Laherty and Bob Eisenman.

Co-immunoprecipitations and Western Blotting

HEK-293 cells were grown as described previously (1) and transfected with pTetOn and pTRE-REST-FLAG using Fugene transfection reagent (Roche Molecular Biochemicals). The tetracycline analogue doxycycline was added to the medium to a final concentration of 2 μg/ml to induce expression of REST. Cells were harvested 48 h after transfection. Co-immunoprecipitation was performed using rabbit anti-Flag (M2) (Sigma, 1:500), goat anti-Myc (Sigma, 1:500), or sheep anti-REST (1:200) antibody. The Flag and Myc epitope-tagged expression constructs using Fugene transfection reagent (Roche Molecular Biochemicals). 48 h after transfection, cells were washed twice in phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde-PBS for 10 min. Cells were then washed four times in PBS, permeabilized for 10 min in 0.1% Triton X-100-PBS (PBST), and incubated for 1 h in 5% nonfat powdered milk in PBST. Coverslips were then incubated for 1 h in an anti-FLAG M5 monoclonal antibody or a polyclonal antibody (Upstate Biotechnology Inc.), both at a 1:500 dilution. Cells were washed four times in PBST before incubating in Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Inc., Eugene, OR) and Alexa 546-conjugated goat anti-rabbit antibody (Molecular Probes), both at a dilution of 1:1000, for 1 h in the dark. Coverslips were then washed four times in PBST and once in PBST and mounted in Vectashield H-1000 (Vector Laboratories, Inc.). Cells were viewed using a Zeiss LSM 510 laser-scanning confocal microscope.

Yeast Reporter Gene Assays

Yeast wild type (WT) and Sin3 deletion strains (DY1641 and DY2516, respectively (12)) were transformed with pMN-REST, pRS313-Sin3 (M1635; Ref. 13), or the corresponding empty expression constructs. Both yeast strains contain an integrated LexA-LacZ reporter so that repression by Lex-R-REST expressed from pMN-REST can be assessed by monitoring β-galactosidase activity.

Mammalian Reporter Gene Assays

HEK-293 cells were grown as described previously (1) and transfected using Fugene (Roche) according to the manufacturer’s instructions. Cells were transfected with 2 μg of the type II sodium channel CAT reporter gene, pSdK7. Cells were also transfected with either 0.5 μg of the “competitor” Gal4P4 or an equimolar amount of the empty Gal4 vector and either 0.5 μg of mSin3A or an equimolar amount of the empty vector pcS2 + MT. Cells were harvested 48 h after transfection. CAT assays were performed as described previously (7).

In Situ Hybridization

Digoxigenin-labeled sense and antisense riboprobes were generated using a digoxigenin RNA labeling kit (SP6/T7; Roche Molecular Biochemicals) and purified on high-performance liquid chromatography columns. Embryos were collected from CD-1 mice at embryonic days 8.5 and 11.5. Whole mount embryos were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS with 2 mM EGTA, “bleached” in 6% H2O2 in PBS with 0.1% Tween 20, and stored at −20 °C in absolute methanol until used for hybridization. Embryos for paraffin embedding were fixed in Bouin’s solution, dehydrated, cleared, embedded in Paraplast, and serially sectioned at 8 μm.

Whole mount hybridization was carried out as described previously (14). Paraffin-sectioned material was hybridized with a modification of the method of Hookfield et al. (15). In brief, sections were deparaffinized, hydrated, proteinase K (10 μg/ml in PBS with Tween)-treated, fixed in 4% paraformaldehyde, acetylated in 0.25% acetic anhydride in 0.1 × trisethanolamine, dehydrated, and dried before hybridization.
mSin3A Is a Functional Co-repressor for REST

FIG. 1. The NH₂ terminus of REST interacts with the NH₂ terminus of mSin3A in yeast. Shown is a yeast two-hybrid assay to test the interaction between full-length mSin3A or the NH₂-terminal half of mSin3A (N-mSin3A) with either the NH₂ terminus of REST (N-REST), the COOH terminus of REST (C-REST), or CoREST. Activity of the β-galactosidase reporter gene is visualized as a blue reaction product in streaks of yeast cells (upper panel). Activity of the His reporter gene enabling growth on medium lacking histidine is indicated by the plus symbol (lower panel). Activity of both reporter genes indicates a positive interaction between two proteins.

The NH₂ terminus of REST interacts directly with the PAH-2 domain of mSin3A. A, GST pull-down assay in which 35S-labeled in vitro translated full-length mSin3A (mSin3A FL) or COOH-terminal deletions to amino acid residues 1015 (mSin3A N1015), 680 (mSin3A N680), 479 (mSin3A 479), or 205 (mSin3A N205) were passed over GST columns. Columns contained either plain GST, GST fused to amino acids 32–122 of REST, and then purified and immobilized on glutathione-Sepharose beads. B, schematic representation of mSin3A constructs used in the GST pull-down experiments. Black filled boxes represent the PAH domains of mSin3A. The ability of each mSin3A constructs to bind GST-REST-(32–122) is indicated as + or −.

RESULTS

The Co-repressor mSin3A Interacts with a Domain in REST Required for Repression—In a yeast two-hybrid screen in which mSin3A was used as the bait, three cDNAs out of a total of 20 sequenced represented REST fragments. Two cDNAs encoded amino acids 32–122 of REST, and one cDNA encoded amino acids 8–135. To test whether the mSin3A interaction was specific for the NH₂ terminus of REST, two LexA-REST fusion proteins were generated, one encoding amino acids 1–525 (N-REST), and the other encoding amino acids 525–1097 (C-REST). The pGAD-mSin3A proteins interacted only with N-REST (Fig. 1). Unfortunately, LexA-full-length REST was not stable in yeast, and therefore we were unable to demonstrate interaction of full-length REST with mSin3A using this system. The mSin3A-interacting domain of REST overlapped with a domain that was shown previously to be required and sufficient for repression of type II sodium channel reporter genes (7). To determine whether mSin3A might also interact with the co-repressor that mediates repression from the COOH-terminal repressor domain of REST, CoREST, further two hybrid analyses were performed (Fig. 1). No interactions were observed between mSin3A or an NH₂-terminal fragment of mSin3A, with either full-length CoREST (Fig. 1) or NH₂-terminal (amino acids 1–283) and COOH-terminal (amino acids 101–482) fragments of CoREST (data not shown).

The mSin3A co-repressor contains four domains that mediate protein-protein interactions, and three of the domains (PAH1–3) are clustered at the NH₂ terminus of the protein. A yeast interaction assay using pGAD N-mSin3A showed that the interaction of REST was also through the NH₂ terminus of mSin3A (Fig. 1). To further characterize the interaction, an in vitro binding assay was performed. A GST fusion protein containing amino acids 32–122 of REST was expressed in bacteria and then purified and immobilized on glutathione-Sepharose beads. In vitro translated mSin3A protein, labeled with [35S]methionine, bound to a GST-retinoblastoma protein col-

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FIG. 3. REST is present in protein complexes precipitated by antibodies to CoREST and mSin3A in mammalian cells. Nuclear extracts from REST-FLAG-transfected HEK-293 cells were immunoprecipitated with antibodies directed against REST, CoREST, the NH₂ terminus of mSin3A (K-20), the PAH-2 domain of mSin3A (AK-11), or preimmune rabbit IgGs (PI). The presence of REST and mSin3A in immunoprecipitates and in 10 μg of the input nuclear extract was examined following SDS-polyacrylamide gel electrophoresis and Western blotting using anti-FLAG and anti-mSin3A antibodies. The migration positions of REST (200 kDa) and mSin3A (150 kDa) are indicated.
umn used as a positive control (16). The radiolabeled mSin3A protein also bound to a GST-REST-(32–122) column but not to GST alone (Fig. 2). Analysis of binding of truncated mSin3A products showed that REST interacts with amino acids 205–479 of mSin3A, corresponding to a region containing the paired amphipathic helix domain 2 (PAH-2; Fig. 2).

**CoREST and mSin3A Form a Complex with REST in Mammalian Cells**—The interactions between REST and mSin3A observed in the yeast two-hybrid assay and in vitro were confirmed in mammalian cells using two approaches. First, co-immunoprecipitation experiments were performed using nuclear extracts from HEK-293 cells overexpressing REST, under control of a tetracycline-inducible promoter. FLAG epitopes were present in immunoprecipitates using REST, CoREST, and mSin3A K-20 antibodies but not in immunoprecipitates using mSin3A AK-11 antibody or preimmune IgG (Fig. 3). Conversely, mSin3A was present in immunoprecipitates using REST, CoREST, and mSin3A antibodies (both K-20 and AK-11; Fig. 3). Given that the AK-11 antibodies recognize the PAH-2 domain in mSin3A, while the K-20 antibodies recognize epitopes in the mSin3A amino terminus, these results are consistent with the GST pull-down experiments above indicating REST interaction through the mSin3A PAH-2 domain.

Second, REST interacted with CoREST and mSin3A overexpressed in intact mammalian cells. In these studies, an epitope-tagged version of REST cDNA lacking a nuclear localization signal (RESTΔNLS-FLAG) was transfected into COS-1 cells. In contrast to WT REST, which is restricted to the nucleus, RESTΔNLS-FLAG was predominantly cytoplasmic, as visualized by immunocytochemistry using an anti-FLAG antibody (Fig. 4). The mutated REST protein caused a dramatic redistribution of both CoREST- and mSin3A-myc fusion proteins to the cytoplasm, whereas they were both strictly nuclear in the presence of WT REST (Fig. 4). Quantitation of the data indicated that mSin3A-myc was redirected to the cytoplasm in 98% of cells expressing RESTΔNLS-FLAG (*n* = 50). Furthermore, the proteins appeared to co-localize within aggregates in the cytoplasm, indicated by the superimposition of the fluorescence.
WT yeast strain, expression of the NH2 terminus of REST fused
mSin3A Is a Functional Co-repressor for REST—The finding
that mSin3A binds to REST through its NH2-terminal repres-
sor domain suggested to us that mSin3A could function as a
corepressor, CoREST, mediated repression from the COOH
terminal of REST (1). In this study, we investigated whether
the known co-repressor mSin3A, a crucial component of many
repressors in mammalian cells, a competition assay was performed using antisense riboprobes specific for mouse mSin3A, REST,
and CoREST. Sense riboprobes were used as controls in each
situation and resulted in no obvious staining (data not shown).

Development—An in situ hybridization study was performed
suppressing the neuronal phenotype outside of the developing
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more restricted pattern of expression, with strong expression in
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DISCUSSION

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REST with mSin3A and CoREST in intact mammalian cells, thus circumventing the potential problem of disruption of complexes during cell extraction procedures. The observation that REST, mSin3A, and CoREST are capable of associating in the cytoplasm indicated that interaction of the co-repressors with REST did not require REST to be bound to its target DNA sequence.

REST and mSin3A were also found to interact in an in vitro "GST pull-down" assay. Furthermore, the combination of the GST pull-down and yeast two-hybrid experiments mapped the sites of interaction on the two proteins. Amino acids 32–122 of REST were sufficient for interaction with mSin3A. Importantly, this 90-amino acid region overlaps with the NH2-terminal domain of REST (amino acids 1–83) previously shown to be required for its repressor activity (7), suggesting that binding of mSin3A is responsible for the repressor activity of this domain. The mSin3A-interacting domain of REST mapped to the region of mSin3A containing PAH-2 and flanking sequences. This was confirmed by the co-immunoprecipitation studies. At present, it is not clear whether the binding of REST to mSin3A is direct or whether it involves other bridging proteins that may be present in the reticulocyte lysate used for the in vitro translation of mSin3A products.

The site on mSin3A through which REST interacts is involved in binding of another DNA-binding protein, Mad (13, 17), and partially overlaps with the site of interaction of the co-repressors SMRT (18, 19) and NCoR (20) and the general transcription factor TFIIB (18). Interestingly, alternatively spliced forms of mSin3B, containing only PAH-1 and PAH-2 and lacking the histone deacetylase interaction domain, have been identified (21). It has been proposed that these shorter forms of mSin3B may attenuate repressor function. Therefore, since REST also appears to bind to PAH-2, it is possible that repression by REST could also be modulated by interaction with shorter dominant negative forms of mSin3. This could provide a way of dynamically regulating the REST repressor complex.

The NH2-terminal repressor domain of REST requires mSin3A for repression. This was most strikingly apparent in yeast, where the ability of the NH2 terminus of REST to repress was abolished in a Sin3-negative yeast strain. In mammalian cells, the ability of endogenous REST to repress reporter genes could be decreased by overexpression of the NH2-terminal repression domain of REST, and this effect could be reversed by co-expression of mSin3A. Although significant, these effects were relatively small, probably due to the presence of the COOH-terminal repression domain of REST.

It has been shown that mSin3A/B interacts with sequence-specific DNA-binding proteins to recruit multiprotein complexes containing histone deacetylases, resulting in the local-
ized deacetylation of histones in the region around specific promoters (22). This is believed to cause nucleosomes to take on a highly condensed structure, preventing access of the transcriptional activation machinery and resulting in transcriptional repression (23, 24). However, it is known that Sin3 proteins are able to mediate repression even in the absence of histone deacetylase activity, suggesting that mSin3A/B utilizes multiple mechanisms for repression (18, 25). In fact, mSin3A is able to bind the general transcription factor TFIIB, suggesting that mSin3A may also repress through direct inhibition of transcriptional initiation (18). Thus, mSin3A-mediated repression through the NH2-terminal repression domain of REST could involve histone deacetylation as reported recently (26) and/or direct inhibition of the transcriptional machinery. Unlike the case for the NCoR and SMRT co-repressors, CoREST did not appear to bind to mSin3A. It remains to be determined whether the two co-repressors utilized by REST converge on components of a common repressor complex or whether the two function by completely independent mechanisms.

REST may utilize mSin3A and CoREST differentially during development. In situ hybridization data showed that at day 8.5 of mouse development mSin3A was expressed fairly ubiquitously, whereas CoREST expression was restricted only to the head region. After this time, by day 11.5, the expression patterns of mSin3A and CoREST were overlapping and extended to most tissues in the embryo. This result suggests either that REST can repress its target genes through recruitment of mSin3A in the absence of a COOH-terminal co-repressor or that other co-repressors can substitute for CoREST at this stage of development. REST repressor complexes may function differently depending on whether they utilize mSin3A alone or in combination with other co-repressors. Future studies to distinguish between these possibilities will provide insight into the processes by which the neuronal phenotype is established and maintained during development.

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