MICoA, a Novel Metastasis-associated Protein 1 (MTA1) Interacting Protein Coactivator, Regulates Estrogen Receptor-α Transactivation Functions*

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The transcriptional activity of estrogen receptor-α (ER-α) is modified by coactivators, corepressors, and chromatin remodeling complexes. We have previously shown that the metastasis-associated protein-1 (MTA1), a component of histone deacetylase and nucleosome remodeling complexes, represses ER-driven transcription by recruiting histone deacetylases to the estrogen receptor element (ERE)-containing target gene chromatin in breast cancer cells. Using a yeast two-hybrid screening to clone MTA1-interacting proteins, we identified a previously uncharacterized molecule, which we named as MTA1-interacting coactivator (MICoA). Our findings suggest that estrogen signaling promotes nuclear translocation of MICoA and that MICoA interacts with MTA1 both in vitro and in vivo. MICoA binds to the C-terminal region of MTA1, whereas MTA1 binds to the N-terminal MICoA containing one nuclear receptor interaction LSRLL motif. We showed that MICoA is an ER coactivator, cooperates with other ER coactivators, stimulates ER-transactivation functions, and associates with the endogenous ER and its target gene promoter chromatin. MTA1 also repressed MICoA-mediated stimulation of ERE-mediated transcription in the presence of ER and ER variants with naturally occurring mutations, such as D351Y and K303R, and that it interfered with the association of MICoA with the ER-target gene chromatin.

Because chromatins is a highly dynamic structure and because MTA1 and MICoA could be detected within the same complex, these findings suggest that MTA1 and MICoA might transmodulate functions of each other and any potential deregulation of MTA1 is likely to contribute to the functional inactivation of the ER pathway, presumably by derecruitment of MICoA from ER target promoter chromatin.

The steroid hormone 17 β-estradiol (E2) plays an important role in controlling the expression of genes involved in a wide variety of biological processes, including reproduction, development, and breast tumor progression (1–3). The biological effects of estrogen are mediated by its binding to the structurally and functionally distinct estrogen receptors (ER-α and ER-β). ER-α is the major estrogen receptor in the mammary epithelium. Like other steroid nuclear receptors, ER-α comprises of an N-terminal transcriptional activation function (AF1) domain, a DNA-binding domain, and a C-terminal ligand-binding domain that contains a ligand-dependent transcriptional activation function 2 (AF2) domain (4). Binding of hormone to ER triggers conformational changes that allow ER to bind the responsive elements in the target gene promoters. The ligand-activated ER-α then translocates to the nucleus, binds to the 19-base pair palindromic estrogen response element (ERE) in the target gene promoters, and stimulates gene transcription, thereby promoting the growth of breast cancer cells. In addition, a series of recent studies also demonstrate other actions of the estrogen receptors, which involve protein-protein interactions (i.e. with AP-1 and SP-1) rather than direct DNA binding.

As with hormonal regulation, the transcriptional activity of ER is affected by a number of regulatory cofactors including chromatin-remodeling complexes, coactivators, and corepressors (5–9). Coactivators generally do not bind to the DNA but are recruited to the target gene promoters through protein-protein interactions with the ER. Examples of ER coactivators include, members of the p160 family, SRC1–3, AIB1, TRAM1, RAC3, cAMP-response element-binding protein-binding protein CBP and p300 (10, 11). Corepressors preferentially associate with antagonist occupied ER (12–14). Among the ER corepressors, NCoR and SMRT are widely characterized molecules that have been implicated in the transcriptional silencing that happens in the absence of ligands (15). In addition, a few bifunctional coregulators such as PELP1 also exist that can act both as coactivators and corepressors of ER (16).

Evidence suggests that multiprotein complexes containing coactivators, ERs, and transcriptional regulators assemble in response to hormone binding and that they activate transcription. The molecular mechanisms of ER, the composition of the ER coactivator proteins, and the way these hormones exhibit tissue or cell type-specific responses are active areas of investigation. A structural analysis of the ER coactivators has identified a 5-amino acid NR motif LXXLL (where X is any amino acid) that can mediate coregulator binding to the liganded ERs (17–19).

For transcription factors to access DNA, the repressive chro-
MATR1 controls the dynamics of ER-driven transactivation by influencing its association with the ER target gene promoter chromatin.

MATERIALS AND METHODS



Plasmid Construction and Two-hybrid Library Screening—The full-length MTA1 (1-715 amino acids) was digested at BamHI and XhoI (blunt end) and ligated to the pGBK7T7 vector that expresses proteins fused to amino acids 1-147 of the GAL4-DNA-binding domain at BamHI and PstI (blunt end) (Clontech). MTA1 baits were constructed by deleting 1-254 amino acids from the N-terminal of MTA1 by cutting and self-ligating with NcoI that cleaves the first 254 amino acids. The remaining 255-715 amino acids of the C-terminal MTA1 were used as bait. This bait was used to screen a mammary gland cDNA library fused to the Gal4 activation domain (Clontech) screened according to manufacturer’s instructions. Positive clones were also verified by one-on-one transformations and selection on agar plates lacking leucine and tryptophan (LT) or adenine, histidine, leucine, and tryptophan (AHLT) and also processed by β-galactosidase assay. Full-length MICOA was either cloned into pCDNA 3.1A or pGEX 5X-1 vectors at EcoRI and XhoI sites.

Cell Cultures and Reagents—Human breast cancer cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum. For estrogen treatment experiments, regular medium was replaced by medium containing 3% DCC (charcoal-stripped serum). Antibodies against c-myc tag were from MBL International, Watertown, MA. Anti-ER was from Upstate Biotechnology, whereas anti-mouse- and anti-rabbit-horseradish peroxydase conjugate were from Amersham Biosciences.

In Situ Hybridization—For in situ hybridization, mouse mammary gland tissues or 13.5-day-old embryos were cut out and fixed with 4% paraformaldehyde and frozen sections were cut (35). In situ hybridization was done by using the digoxigenin (Roche Diagnostics)-labeled riboprobe. A 375-bp mouse MICOA cDNA was amplified by reverse transcriptase-PCR, subcloned into TOPO II vector (Promega), and used for riboprobe synthesis under the control of T7 promoter. Primers used were: F, CCACCGCGGATTTCGTTCCGCTCCGGCTTC; R, GGAGG-GAACCTGACAGCTGAGGAGAGCACAGA. RNA probes were labeled with digoxigenin and hybridized for 16–20 h in buffer containing 1 μg/ml riboprobes, 50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.4), 10 mM NaH2PO4 (pH 6.8), 5 mM EDTA (pH 8.0), 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 10% dextran sulfate, 200 μg/ml yeast total RNA, and 50 mM diethyldithitol. Alkaline phosphatase-labeled sheep anti-digoxigenin antibody was applied and signals were visualized by nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate. Hybridization with sense probe was used as background control.

Chromatin Immunoprecipitation Assay—Approximately 106 cells were treated with 1% formaldehyde (final concentration, v/v) for 10 min at 37 °C to cross-link histones to DNA. Chromatin immunoprecipitation assay was performed as described (35). The sequences of the forward and reverse primers for pS2 used in this study were: GAATTACGTTAGGCCCTAGGCAAGGTAAG and AGGATTTGCTGATGACAGAGGACGC, respectively.

HAT Assay—Cells were either treated with/without estrogens (10−8 M). Then cells were lysed and immunoprecipitated with anti-T7 antibody. Immunoprecipitate was taken for histone acetyltransferase assay by the HAT-Check activity assay kit (Pierce). HAT assay with positive control in each assay was performed as per the instructions with little modifications (5).

Immunofluorescence and Confocal Imaging—MCF7 cells were plated on glass coverslips in 6-well culture plates. When the cells were ~50% confluent, they were changed to DCC medium supplemented with 5% fetal calf serum for 48 h, then treated with estrogen (10−8 M) for 30 min with or without pretreatment with the anti-estrogen ICI (10−8 M) for 1 h. Cells were rinsed with phosphate-buffered saline, fixed in cold methanol for 6 min, then processed for immunofluorescence staining of c-estrogen receptor. Cells were counterstained with ToPro3 to visualize the nucleus. Slides were further processed for imaging and confocal analysis using a Zeiss LSM 510 microscope and a ×40 objective.

Transfection and Promoter Assays—Cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (1:1) supplemented with 10%.
fetal calf serum. For reporter gene transient transfections, cells were cultured in medium without phenol red and containing 3% charcoal-stripped (DCC) serum for 24–36 h, and promoter assays and in vitro transcription and translation and glutathione S-transferase (GST) pull-down assays were performed as previously described (35).

RESULTS

Identification of MICoA as MTA1-interacting Protein—MTA1 is expressed in a wide range of tissues, yet the nature of its downstream targets remains unknown. To identify novel MTA1-interacting proteins, we performed a yeast two-hybrid screening of the mammary gland cDNA expression library using MTA1 C-terminal amino acids 255–715 and MTA1 N-terminal amino acids 1–254 as the baits. As a negative control, we used the recently identified MTA1s variant that lacks protein-binding motifs (38) as a bait. Yeast cells expressing the Gal4 fusion protein were transformed with the above bait. Screening of 2 × 10^6 transformants resulted in the isolation of several positive clones. Sequencing of the positive clones identified several clones that encoded the full-length cDNA of a previously uncharacterized gene assigned to chromosome 12q13-q14 (GenBank™ accession number S82447). To further confirm the interaction of MICoA with MTA1, we cotransfected C-terminal or N-terminal MTA1 constructs with MICoA and the transformed colonies showed both the ability to grow in medium lacking adenosine, histidine, tryptophan, and leucine (AHTL) and to turn blue in a β-galactosidase assay, whereas cotransfection with the control GBK vector did not do so (Fig. 1A).

Because we discovered a coactivator function of this gene product (see below), we named this protein MICoA. MICoA contains a total of 378 base pairs and encodes a protein of 125 amino acids (about 14 kDa) and has an overall 23.5% scattered homology with yeast GCN5. Sequence analysis of MICoA revealed the presence of a consensus nuclear receptor binding site (NR box, LXXLL) in the N-terminal region and a casein kinase phosphorylation site (TALE) in the C-terminal segment (Fig. 1B).

MICoA mRNA was easily detectable in ER-positive breast cancer cell lines, such as MCF-7, ZR-75R, and T47D but modest expression levels could also be detected in other cell lines (Fig. 1C). Full-length MICoA has been cloned into pCDNA3.1A and pGEX 5X-1 vectors. Their expression has been shown in Fig. 1D.

MICoA Expression during Development—To gain clues about the possible functions of MICoA in vivo, we examined the expression of MICoA mRNA in multiple mouse tissues. As shown in Fig. 2A, MICoA mRNA could be easily detected in many mouse tissues, with the highest level in the mammary glands of pregnant mice. To get a deeper view, we further performed in situ hybridization to determine the levels of MICoA expression during embryonic development and in the mammary glands. At the 13.5 day mouse embryo, MICoA was widely expressed in various tissues (Fig. 2B). In mouse mammary glands, MICoA expression was seen in ductal epithelial cells as well as in fat cells, whereas the expression became much stronger in the alveolar cells during pregnancy (Fig. 2C).

MICoA Interacts with MTA1 in Vitro and in Vivo—To con-
Corepressor-interacting Coactivator in ER Functions

Direct association of MICoA with MTA1.

A. MICoA directly interacts with MTA1. In vitro translated T7-MICoA protein was incubated with GST-MTA1 or GST alone, and GST pull-down assays were performed, to show the association of MTA1 with MICoA (n = 3). B, ZR-75 cells were transiently transfected with T7-MICoA and c-myc-MTA1 or a control vector and treated with or without E2 (10^{-9} M) for 16 h. Immunoprecipitation was performed using c-myc-mAb, and Western blotting was performed with the anti-T7 antibody (n = 3). C, ZR-75 cells were transiently transfected with T7-MICoA and c-myc-MTA1 or a control vector and treated with or without E2 (10^{-9} M) for 30 min. Confocal images of single optical sections are shown. Double labeling was performed with a rabbit antibody against Myc for MICoA (visualized in green) and a mouse monoclonal antibody against T7 for MTA1 (visualized in red). E2 treatment induced colocalization of the T7-MICoA with myc-MTA1 in large nuclear domains, as shown by the yellow (green and red combined) in the merged images.

MICoA Acts as a Coactivator of ER-α—The presence of an LSRRLL motif and the abundant expression of MICoA in the mammary glands of pregnant mice raised the possibility of the role of MICoA in the ER pathway. To explore this notion, we examined the potential ability of MICoA to influence the transcription from an ERE-luciferase reporter system, using either ER-positive ZR-75 or MCF-7/LTED cells or ER-negative HeLa cells co-transfected with the ER. Co-expression of MICoA resulted in more stimulation of ERE-driven transcription in ZR-75R cells treated with E2 treatment than in the mock-treated cells (Fig. 5A). The expression of MICoA alone in hypersensitive MCF-7/LTED cells (39) had a modest but reproducible stimulatory effect on reporter activity in the absence of ligands. This MICoA-mediated increase in the ERE-luciferase activity was dose-dependent, with the highest activity at 500 ng of DNA (Fig. 5B). As with other well characterized coactivators (Fig. 5C), cotransfection of HeLa cells with PELP1 and T7-MICoA but not vector control significantly increased ERE-luciferase reporter activity by PELP1, a recently identified ER coactivator. Because the LXXLL motif is also important for binding to other nuclear receptors we tried to explore the effect of MICoA on progesterone receptor as well as glucocorticoid receptor by using PRE-luciferase and GRE-CAT (Fig. 5, D and E). MICoA could induce progesterone receptor transactivation, whereas glucocorticoid receptor transactivation remains unaltered. Together, these findings suggest that MICoA is a coactivator of the ER pathway and selective in its transactivation function.

MICoA Associates with the ERE-containing Promoters in Vivo—To directly demonstrate the potential importance of MICoA in ERE transcription, we used the chromatin immunoprecipitation assay to analyze whether T7-MICoA associates with the endogenous ERE-containing promoters. MCF-7 cells were transiently transfected with T7-MICoA and Myc-MTA1. The cells were grown in phenol red-free medium supplemented with 3% charcoal-stripped serum, treated with E2 (10^{-9} M) for 30 min, and then fixed in methanol and stained for c-myc tag (green) and T7-tag (red), and counterstained for nuclear DNA (blue). As shown in Fig. 2C, T7-MICoA was localized predominantly in the cytoplasm, but upon E2 stimulation it was redistributed to the nucleus suggesting that this effect is mediated by the ER, and could be blocked by anti-estrogen ICI 182780 (data not shown). Areas of colocalization of the T7-MICoA and c-myc-MTA1 proteins resulted in the development of yellow fluorescence because of the merging of the red and green pixels (Fig. 3C). Transfected MTA1 was primarily localized in the nucleus. These findings suggest that E2 signaling promotes the nuclear translocation of MICoA.

MICoA Interacts with the C-terminal Region of MTA1—Next, we defined the minimal region of MTA1 required for its interaction with MICoA. MTA1 has several important domains involved in protein-protein interactions, DNA binding, and signaling (Fig. 4A). Several C-terminal MTA1 deletion constructs were generated and expressed as 35S-labeled proteins; they were then subjected to GST pull-down assays with the GST-MICoA fusion protein. Results suggest that amino acids 441-703 of MTA1, which contains one SH3 and one SH2 site, constituted the binding region for MICoA (Fig. 4B, left panels). To define the binding region or regions of MICoA that are important for MTA1 interaction, we disrupted the NR box by deleting the LSRLL motif (MICoA-del-1). In addition, we also deleted the CK2 phosphorylation consensus site (MICoA-del-2) or both the NR box and CK2 phosphorylation sites (MICoA-del-3) (Fig. 4C). Results of the GST pull-down assays indicate that MICoA used its LSRRLL motif to interact with MTA1 (Fig. 4D). This further demonstrates that the N-terminal region of MICoA interacts with the C-terminal region of MTA1.

E2 signaling promotes the nuclear translocation of MICoA.

MICoA interacts with the C-terminal region of MTA1. To demonstrate the association of MICoA with MTA1, we next examined the ability of in vitro translated MICoA protein to bind with GST-MTA1 in vitro. MICoA interacted efficiently with GST-MTA1 but not with GST alone in GST pull-down assays (Fig. 3A). To demonstrate the interaction of MICoA and MTA1 in breast cancer cells, the ZR-75R breast cancer cells were co-transfected with T7-tagged MICoA, c-myc-tagged MTA1, or a pCMV control vector. Immunoprecipitation of cell lysates with an anti-T7 monoclonal antibody was followed by immunoblotting with the anti-c-myc antibody. Results showed a specific baseline as well as an estrogen (E2)-inducible interaction between the T7-MICoA and c-myc-MTA1 (Fig. 3B). We explored the spatial relationship between MICoA and MTA1 within cells using immunofluorescence and confocal scanning microscopy. ZR-75 cells were transiently transfected with T7-MICoA and Myc-MTA1. The cells were grown in phenol red-free medium supplemented with 3% charcoal-stripped serum, treated with E2 (10^{-9} M) for 30 min, and then fixed in methanol and stained for c-myc tag (green) and T7-tag (red), and counterstained for nuclear DNA (blue). As shown in Fig. 2C, T7-MICoA was localized predominantly in the cytoplasm, but upon E2 stimulation it was redistributed to the nucleus suggesting that this effect is mediated by the ER, and could be blocked by anti-estrogen ICI 182780 (data not shown). Areas of colocalization of the T7-MICoA and c-myc-MTA1 proteins resulted in the development of yellow fluorescence because of the merging of the red and green pixels (Fig. 3C). Transfected MTA1 was primarily localized in the nucleus. These findings suggest that E2 signaling promotes the nuclear translocation of MICoA.
transfected with T7-MiC0A were treated with E2 for different lengths of time and processed for formaldehyde cross-linking. Sonicated chromatin was subjected to immunoprecipitation with specific antibodies against T7. T7-MiC0A-bound genomic DNA fragments were analyzed by quantitative PCR using primers spanning the ERE present in the promoter of the pS2 sequence, for a potential E2-triggered association of T7-MiC0A with the promoter of the ER target gene. Results indicated that E2 treatment triggered a significant increase in the amount of pS2 (12-fold more than for untreated cells) target gene promoter chromatin associated with T7-MiC0A (Fig. 6A). Because MiC0A promoted transcription from the ERE containing promoter (Fig. 5) and interacted effectively with the ER target gene chromatin (Fig. 6A), these findings raised the possibility that MiC0A may influence the status of chromatin remodeling, presumably through HAT activity. Therefore, we next explored whether such activity is associated with the T7-MiC0A complex. Immunoprecipitation of T7-MiC0A from MCF-7 cells showed the presence of functional HAT activity with the MiC0A complex (Fig. 6B). Because there is no HAT domain in the MiC0A, the HAT activity we detected is likely to come from the proteins that could associate with MiC0A in vivo. In brief, these findings strongly support the notion that MiC0A influences the status of the ER target gene promoter using chromatin-remodeling mechanisms.

MTA1 Represses the Stimulation of MiC0A-induced Transcription from ERE—Because MTA1 acts as a corepressor of the ER pathway (35) and because MiC0A is both an ER coactivator and an MTA1-interacting protein (as demonstrated in this study), we next investigated the potential impact of MTA1 deregulation on MiC0A-mediated stimulation of ERE transcription. The ZR-75 breast cancer cells were co-transfected with ERE-luciferase, MiC0A, and MTA1 or with a control vector, and then stimulated with E2. Coexpression of MTA1 suppressed both MiC0A-stimulated and E2-induced ERE-driven transcription (Fig. 7A). Similar results were obtained when we used the MCF-7 cells stably expressing T7-MTA1 (Fig. 7B). We explored the possibility of neutralizing the ER corepressor MTA1 by MiC0A using MCF-7 cells stably overexpressing PELP1 coactivator (40). As shown in Fig. 6C, MiC0A was a potent activator of ER transactivation in MCF-7/PELP1 cells. In addition, we also noticed that E2 treatment promoted interactions of T7-PELP1 with the endogenous MTA1 (Fig. 7C, inset). Furthermore, MTA1 overexpression also repressed the transcription normally stimulated by the coexpression of MiC0A and PELP1 in MCF-7 cells (Fig. 7C, last two sets of columns). Because MTA1 and MiC0A proteins have opposing effects on ERE transcription, our finding suggests that the MTA1 corepressor activity is dominant over the coactivator function of MiC0A.

Effect of MiC0A on Transactivation Functions of ER with Naturally Occurring Mutations—In recent years, several naturally occurring mutations have been found in the ERs of breast cancer cells. One mutant ER, D351Y in the ligand-
binding domain, was found in a tamoxifen-stimulated tumor line and enhances the estrogen-like actions of tamoxifen and raloxifene (12, 41–44). Breast cancer cells with mutant ERs exhibit differential responsiveness to estrogen and anti-estrogen and this phenomenon may be associated with the potential differential recruitment of nuclear coregulators (42, 44). To explore the role of coregulators in the actions of mutant ERs, we next determined the ability of MICoA, MTA1, or both to modulate the ER-transactivation function. As expected, expression of K303R ER in HeLa cells resulted in hypersensitivity to the E2 response compared with expression of the wild-type ER (Fig. 8A). Interestingly, coexpression of MICoA was accompanied by a further enhancement of the E2 response compared with the levels of ERE-driven transcription activated by the individual expression of K303R ER or MICoA. However, MTA1 overexpression effectively blocked the stimulation of ERE transcription by the mutant K303R ER in both the presence and absence of MICoA (Fig. 8A). To examine the effect of MICoA on the mutant D351Y ER, we used well characterized MDA-MB231 breast cancer cells stably expressing either wild-type ER (S30 cells) or D351R ER (BC cells) or D351G (JM cells) (45). As shown in Fig. 7B, MICoA stimulated ERE transactivation to a comparable extent in S30 as well as BC, but not in JM cell lines, and this stimulatory action was repressed by the overexpression of MTA1. In brief, these findings suggest that MICoA and MTA1 have potent influence on both ERs, naturally existing mutant ER, or mutant ER mimicking acetylated ER. The transactivation function is lost probably when it cannot bind to mutant ER (JM cells).

**MICoA Interacts with the ER-α**—Because MICoA functions as a coactivator of ER, we next determined whether MICoA interacts with ER by using GST pull-down assays with the full-length or deletion mutants of the ER. Results indicated that GST-MICoA but not GST alone bound to the C-terminal region (amino acids 301–552) containing the AF2 domain of the ER as well as 264–300 amino acids representing the hinge region (Fig. 9, A and B). Because MICoA interacts with the AF2 domain of the ER (Fig. 9, A and B), we next tested the significance of this interaction by examining the recruitment of MICoA complexes to ER elements using a Gal4-ER/Gal4-luciferase assay system (46, 47). This system involves transient transfection of two Gal4-AF2 plasmids (ligand-binding domain of ER-α and Gal4-luciferase reporter), and luciferase activation depends on E2 stimulation of the AF2 domain. In this assay the E2-mediated activation of the AF2 function was further stimulated by MICoA expression (Fig. 9C). Many coactivators have
been shown to activate the transcription of specific promoters when recruited by a heterologous DNA-binding domain. In brief, these results suggested that MICoA-ER interaction might play a role in the coactivator function of MICoA.

Coactivator Function of MICoA Requires LSRLL Motif—To explore the mechanism of MICoA regulation of ER transactivation, we investigated the significance of the nuclear receptor-binding motif in MICoA. As illustrated in Fig. 10, MICoA with a mutated LSRLL motif was not able to stimulate ERE transcription in response to E2 treatment and exhibited much lower baseline ER transactivation activity. In addition, the MICoA without the mutated NR box motif did not bind to the GST-AF2 as opposed to binding of wild type MICoA with GST AF2 (Fig. 10). Because E2 stimulation triggered a rapid redistribution of T7-MICoA from the cytoplasm to the nucleus, we next determined the potential role of LSRLL in the nuclear translocation of MICoA. Interestingly, the MICoA with mutated LSRLL motif failed to translocate to the nucleus in E2-stimulated cells (Fig. 10). Together, these results suggest that the LSRLL motif plays an important role in the movement of MICoA to the nucleus, binding of MICoA with estrogen receptor, and the ER transactivation function of MICoA.

MTA1 Modifies MICoAs Association with Target Chromatin—To understand the physiological significance of MICoA-MTA1 interaction in vivo, we determined whether MTA1 overexpression might influence the ability of MICoA to associate with the ER target gene promoter pS2. Breast cancer cells were cotransfected with T7-MICoA or T7-pcDNA and were treated with or without E2 \((10^{-9}\text{M})\) for 3 h. Cell lysates were immunoprecipitated with antibodies against T7, and the associated HAT activity was determined as described under "Materials and Methods." MTA1 coexpression reduced this association of T7-MICoA with the pS2 promoter chromatin (Fig. 11A, lane 6). Because MTA1 physically associates with HDAC2 (35), our findings suggest that MICoA associates with the HDAC2 that is associated with MTA1. Indeed, the coimmunoprecipitation assays demonstrated the association of T7-MICoA with HDAC2 in the presence of c-myc-MTA1 and showed that E2 stimulation impaired the interaction of HDAC2 with the MICoA-MTA1 complex (Fig. 11B).

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There was no association of HDAC2 with T7-MICOa in the absence of c-myc-MTA1 (data not shown). Results from the coimmunoprecipitation assays also show that T7-MICOa interacted effectively with the endogenous ER-
H9251, and that MTA1 overexpression prevented such interactions (Fig. 11C, compare lane 4 with lane 2). Together, these results suggest that the coactivator functions of MICoA are closely linked with the ability of MICoA to interact with the ER and to recruit such a complex to the target gene promoter chromatin; they also suggest that MTA1 overexpression represses MICoA regulation of the ER pathway.

**DISCUSSION**

The NR superfamily of transcription factors regulates transcription activity upon the binding of specific steroid hormones (48–50). A conserved amphipathic α-helical structure within the AF2 region of these factors is required for their ligand-dependent transcriptional activation, whereas another region known as activation domain 1 is responsible for their ligand-independent transactivating activity. It is increasingly accepted that NRs require a series of coregulators (i.e. coactivators and corepressors) for optimal transcription activity (51–55). Because several of these corepressors and coactivators have either intrinsic or associated HDAC and HAT activity, respectively, it has been proposed that transactivating functions of the ER, and of the NR in general are regulated by the combined action of coregulators and the stage of the chromatin-remodeling process. Because the target gene promoter chromatin is a highly dynamic structure, the transactivating functions of coactivators are likely to be influenced by corepressors and any potential deregulation of one component will have functional implications on the action of other components. However, to date this notion has not been validated for ER in breast cancer cells. In the current study we found that a corepressor can co-exist with a co-activator and the functional manifestation is dependent upon the interplay of several other coregulators. This could be an important way of regulating ER transactivation in breast cancer cells.

The interaction of MICoA with MTA1 has been mapped to the region of MTA1 having the binding sites for SH3 and SH2. There is a possibility that MICoA might be competing with the proteins possessing SH3 or SH2 domains. Interestingly, a newly discovered variant of MTA1, the MTA1s showed no interaction with the MICoA, suggesting that MICoA is a specific binding protein for the MTA1.

The results of our study show that the MICoA is an MTA1-interacting protein and that MICoA interacts with the endogenous ER, stimulates the ER transactivation function, and associates with the ER target gene promoter chromatin, presumably by recruiting HATs. MICoA is a novel ER coactivator with an overall 23% scattered homology with yeast GCN5L1 (56).
MICoA does not contain a HAT motif, the HAT activity is likely to come from its association with other interacting proteins. Structural and functional analyses of several coactivators revealed that coactivators interact with the ligand-bound AF2 domain through the LXXLL motif and are sufficient to mediate the binding of coactivators to ligand-bound NRs. A single LXXLL motif is enough to allow activation of ER by E2. MICoA is distinct from other coactivators in that it is localized primarily in the cytoplasm and is translocated to the nucleus upon E2 signaling. Furthermore, MICoA directly interacts with the AF2 domain of the ER, and binding may be enhanced by E2 signaling. In addition, like other coactivators, MICoA exhibits an added stimulation of ER transactivation functions in the presence of other coactivators, such as PELP1 (Fig. 5C).

The mechanism by which MICoA activates ERE transcription is not fully understood but appears to involve an absolute requirement of the LSRLL motif in both the cytoplasm-to-nucleus redistribution of MICoA and the transactivation function of ER. However, despite the presence of the LSRLL motif, MICoA did not sequester ER in the cytoplasm. This finding and the fact that E2 signaling is required to activate the MICoA suggest that E2 signaling either modifies the protein-protein interaction of MICoA via the LSRLL motif or alters the conformation of MICoA to favor its nuclear translocation. In addition, it is also possible that estradiol causes an effect to block a nuclear export process so that one disrupts the equilibrium between the cytoplasm and nucleus that results in MICoA being largely in the nucleus after exposure to E2. The LXXLL motifs are important in mediating the protein-protein interactions between SRC1-3 and p300/CPB (18). It is also possible that the small amount of ER present in the cytoplasm is sufficient for the formation of the MICoA-ER complex upon E2.
stimulation and subsequent translocation of MICoA to the nucleus. Upon deletion of the LSRLL motif MICoA lost its ER transactivation function indicating that this motif is important for the said function. Alternatively, it is also possible that the translocation of MICoA to the nucleus is facilitated by the bound MTA1, and that MICoA needs the presence of MTA1 to act like a coactivator. Furthermore, MICoA could also act as an antagonist of a repressor function of MTA1 thereby functioning as a coactivator. Clearly, additional studies are needed to address these evolving questions.

Our finding that the ER coactivator MICoA interacts with MTA1, an ER corepressor, is surprising, as it raises the possibility that the final outcome of the ER transactivation function is influenced by complex protein-protein interactions rather than by isolated interaction with one class of proteins. It has been proposed that different HDAC complexes such as the mSin3 complex are recruited for simple deacetylation of dynamically regulated promoters, whereas nuclear remodeling and deacetylation and CoREST-HDAC complexes are required for promoters that require stable repression (e.g. tissue specific silencing) (44). The associated non-enzymatic activities may play a role in determining the nature of the repression. From our results, it appears that MTA1 has inhibitory activity against MICoA-mediated interaction with ER and stimulation of ER transactivation. A modest but significant reversal of MTA1-MICoA recruitment on one of the E2 target genes, i.e. pS2 promoter, was achieved by E2 stimulation of cells and was accompanied by the loss of HDAC2 interaction with the MICoA-MTA1 complex (Fig 11). In addition, our results from the binding studies suggest that the LXXLL motif in MICoA is important for its binding to both MTA1 and ER-α (Figs. 4, C and D, and 10). Because the presence of MTA1 influenced the recruitment of MICoA to the ER target gene promoter (pS2 promoter), these results implied an ongoing potential competition between ER and MTA1 to interact with MICoA and thus, affecting the recruitment of MICoA on ERE of the target gene promoter. We also found out that the ER association with MICoA is abrogated when MICoA interacts with MTA1 complex. Because MTA1 exhibited an overall corepressor function in the presence of MICoA and E2 stimulation, these findings suggested that HDAC2 association with MICoA might not constitute a major mechanism of MTA1-mediated corepression of MICoA-mediated ERE activation. Modest but distinct withdrawal of MICoA from the pS2 promoter in the presence of MTA1 and dismissal of ER from the complex could be the reason for the corepressive function of MTA1 taking over the coactivator function of MICoA. Recently Nye et al. (57) have reported about the large scale chromatin unfolding activity by estrogen receptor.

Physiological significance of our findings is based on the observation of the association of MTA1 and MICoA with the ER target gene promoter chromatin. Because the level of MTA1 is up-regulated in breast cancer cells and by heregulin signaling (35), our current findings imply a potential inhibition of the coactivator functions of MICoA by the pathologic level of MTA1 and suggest that these events may modulate the hormonal response in breast cancer cells. Particularly important is the ability of MICoA to further induce the transactivation of the natural ER mutant (D351Y) found in tamoxifen-resistant breast cancer cells. This finding suggests that MICoA is not only a potent coactivator of ER but may also be an important regulatory molecule in the context of breast cancer. MICoA could not further induce asparatate at the 351 position, when replaced by charge-less glycine. It is reported that D351G loses the ability to bind to coactivator because of charge-less glycine (45). Such mutation could have interfered with binding of MICoA with the mutant ER. This further says that the transactivation function for MICoA of the ER is probably dependent upon its physical interaction with ER. In another instance MICoA could further induce the transactivation function of hypersensitive ER-α (K303R) in the presence of E2. A similar finding was reported for p300, which has histone acetyltransferase activity of its own (43). This could be attributed to the HAT activity present in the MICoA complex on E2 signaling. The interaction of MICoA with the hinge domain of ER might also be instrumental for such effect on K303R ER.

In summary, the present study identified MICoA as a target of corepressor MTA1, established the coactivator function of MICoA, and provided new evidence to suggest that the transactivation functions of ER are influenced by the regulatory interactions between MICoA and MTA1. Our finding is in agreement with the emerging model involving coactivator and corepressor in the same complex, implying that MTA1 and
MIcAo might transmodulate functions of each other and that any potential deregulation of MTA1 is likely to contribute to the functional inactivation of the ER pathway, presumably by derecruitment of MIcAo from the ER target promoter chromatin.

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Corepressor-interacting Coactivator in ER Functions

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MICoA, a Novel Metastasis-associated Protein 1 (MTA1) Interacting Protein Coactivator, Regulates Estrogen Receptor-α Transactivation Functions
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