The Coactivator activator CoAA regulates PEA3 group member transcriptional activity

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The PEA3 (polyoma enhancer activator 3) group members [ERM (ETS-related molecule), ER81 (ETS-related 81) and PEA3] of the Ets transcription factor family are involved in migration and dissemination processes during organogenesis and cancer development. In the present study, we report that the hnRNP (heterogeneous nuclear ribonucleoprotein)-like protein CoAA (Coactivator activator) interacts with the PEA3 group members and modulates their transcriptional activity. We also demonstrate that the CoAA YQ domain, containing tyrosine/glutamine-rich hexapeptide repeats, is necessary for the interaction, whereas the two N-terminal RRMs (RNA recognition motifs) of CoAA are required to enhance transcriptional activity. Finally, we show that CoAA is involved in the migration-enhancing action of PEA3 on MCF7 human cancer cells, suggesting that CoAA might be an important regulator of PEA3 group member activity during metastasis.

Key words: Coactivator activator (CoAA), ETS-related molecule (ERM), Ets transcription factor, migration, polyoma enhancer activator 3 (PEA3), RNA recognition motif (RRM), transcriptional regulation.

INTRODUCTION

The Ets transcription factors share a DNA-binding domain (the ETS domain) of approximately 85 residues that binds the consensus core sequence 5’-GGAA/T-3’ (reviewed in [1]). On the basis of variations within this domain, different groups are distinguished [1]. The PEA3 (polyoma enhancer activator 3) group is composed of three highly conserved Ets transcription factors ERM (ETS-related molecule), ER81 (ETS-related 81) and PEA3. These factors are often overexpressed in cancers with a disseminating phenotype, and experimental modulation of their expression influences the invasive process, suggesting a key role for these factors in metastasis (reviewed in [2,3]). For example, PEA3 expression is sufficient to confer an invasive phenotype on MCF7 human cancer cells [4]. Moreover, PEA3 and ERM have been described as factors of poor prognosis in ovarian and breast tumours respectively [5,6]. PEA3 group members are prone to post-translational modifications, such as phosphorylation, acetylation, ubiquitinylation and SUMOylation [7–14] that regulate their transcriptional activity. Their activity is also regulated by interaction with protein partners such as Jun, USF-1 (upstream stimulatory factor-1) and CBP (CREB (cAMP-response-element-binding protein)-binding protein)/p300 [13,15,16].

CoAA (Coactivator activator; RBM14) was originally reported as an activator of the coactivator TRBP (thyroid-hormone-receptor-binding protein) [17]. It was also isolated as a partner of a proto-oncogene, SYT (synovial sarcoma translocation protein), a nuclear receptor coactivator known to bind to the SWI/SNF chromatin-remodelling complex [18,19]. Recently, Li et al. [20] have shown that CoAA interacts with the RUNX2 DNA-binding domain, preventing RUNX2-driven gene expression. CoAA is an hnRNP (heterogeneous nuclear ribonucleoprotein)-like protein containing two N-terminal RRMs (RNA recognition motifs) enabling it to act as a regulator of mRNA splicing [21–23]. The central YQ domain of CoAA required for its interaction with TRBP, SYT and RUNX2 possesses 27 tyrosine/glutamine-rich hexapeptide repeats and exhibits transcription-regulating activity [17,18]. Such a domain is also present in the oncoproteins EWS (Ewing’s sarcoma), TLS/FUS (translocation/fusion in liposarcoma) and SYT [18,24]. CoAA is widely expressed in embryonic tissues, and its expression is prone to alternative splicing [25,26]. For example, splicing events can result in expression of the CoAM (Coactivator modulator) splice variant. The balance between CoAA and CoAM isoforms has been clearly implicated in the regulation of early embryonic development [25,26]. Furthermore, CoAA is overexpressed in a variety of cancers, its overexpression in NIH 3T3 cells increases cell proliferation and promotes colony formation in soft agar assays [24], whereas its knockdown blocks osteosarcoma cell growth in vitro [20]. Paradoxically, underexpression of CoAA transcripts is observed in certain cancers such as renal carcinoma. In renal carcinoma cells, it has previously been demonstrated that CoAA can act as a tumour suppressor by repressing expression of the c-Myc proto-oncogene [27]. CoAA can thus increase or decrease cell proliferation, depending on the cell model.

Abbreviations used: CBP, CREB (cAMP-response-element-binding protein)-binding protein; CMV, cytomegalovirus; CoAA, Coactivator activator; CoAM, Coactivator modulator; DTT, dithiothreitol; ER81, ETS-related 81; ERM, ETS-related molecule; EWS, Ewing’s sarcoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; GHBP, GH-binding protein; GST, glutathione transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule-1; MMTV, murine mammary tumour virus; PEA3, polyoma enhancer activator 3; qPCR, quantitative PCR; RRM, RNA recognition motif; RT, reverse transcription; siRNA, small interfering RNA; SYT, synovial sarcoma translocation protein; TLS/FUS, translocation/fusion in liposarcoma; TRBP, thyroid-hormone-receptor-binding protein.

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In the present study we have used biotinylated ERM as bait to fish for new potential ERM partners in nuclear extracts and identified CoAA. We show that endogenous ERM and CoAA interact both in vitro and within cells, and that CoAA modulates ERM transcriptional activity by interacting with ERM via its YQ domain. Interestingly, the results of the present study also indicate that the CoAA RMs are required to enhance ERM activity. Similar effects are observed for the two other PEA3 group members PEA3 and ER81. Moreover, we show that knockdown of CoAA decreases ERM- or PEA3-induced activation of ICAM-1 (intercellular adhesion molecule-1). Finally, we demonstrate that in MCF7 human cancer cells, the PEA3 migratory activity is enhanced upon CoAA overexpression and decreased upon CoAA knockdown.

EXPERIMENTAL

Plasmid constructs

The pSG5 expression vectors encoding full-length ERM, ER81 or PEA3 have been described previously [12,14,28,29]. The FLAG-tagged ERM vector (pSV FLAG-ERM) is described in [11]. The GST (glutathione transferase) fusion protein expression vectors have also been described previously [30,31]. The vectors expressing FLAG-tagged CoAA, CoOM or AxxQ were provided by Dr L. Ko (Medical College of Georgia, Institute of Molecular Medicine and Genetics, Augusta, GA, U.S.A.) [17,24]. The vectors expressing Myc-tagged CoAA, CoAAC, A1NCoAAC or hnrRNA1 were a gift from Dr D. Auboeuf (INSERM U685, Institut Universitaire d’Hématologie, Hôpital Saint-Louis, Paris, France) [22]. The pDelta-EndoA-Luc (EndoA-luciferase) plasmid carries a trimeric synthetic repeat of the EndoA enhancer and was a gift from Dr Iniguez-Lluhi (University of Michigan Medical School, Ann Arbor, MI, U.S.A.) [32]. The ICAM1–Luc, CMV (cytomegalovirus)–β-galactosidase, E2F1–Luc and 5xE2F–Luc reporter plasmids have been described previously [14,28,33].

Isolation of CoAA

After washing in PBS, HeLa cells were lysed in hypotonic buffer [10 mM Hepes/KOH (pH 7.9), 10 mM KCl, 3 mM MgCl2, 1 mM DTT (dithiothreitol), 0.1 % Nonidet P40 and 1× Complete™ protease inhibitor (Roche)] for 5 min on ice. After centrifugation (1000 g for 15 min at 4°C), the cytoplasmic supernatant was removed and the remaining nuclear pellet was resuspended in benzonase buffer [20 mM Hepes (pH 8), 150 mM NaCl, 3 mM MgCl2, 1 mM DTT, 0.1 % Nonidet P40 and 1× Complete™ protease inhibitor (Roche)], after which benzonase digestion was performed for 2 h at 4°C. After centrifugation at 30,000 rev./min for 1 h at 4°C (rotor type SW41Ti, Beckman), the supernatant was incubated for 2 h at 4°C with biotinylated ERM (produced in Escherichia coli) and purified on streptavidin-coupled beads; the detailed procedure is available upon request). The beads were then washed five times with benzonase buffer containing 250 mM NaCl and twice with benzonase buffer. The captured proteins were then eluted in Laemmli buffer, separated by SDS/PAGE, stained with Coomassie Brilliant Blue R-250, excised from the gel, and identified by MS as described previously [34].

In vitro protein–protein interaction

[35S]Methionine-labelled proteins were produced with the TnT® T7 Quick Coupled Transcription-Translation System (Promega). The GST-fusion proteins or GST alone bound to glutathione beads were incubated for 2 h at room temperature (20°C) with the in-vitro-translated proteins in binding buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % Triton X-100, 40 μg/ml BSA and 1 mM DTT. After extensive washing with the same buffer, the bound proteins were analysed by SDS/PAGE and revealed by autoradiography.

Cell culture and transfection

RK13 rabbit kidney cells, U2OS human osteosarcoma cells, HeLa human cervical cancer cells, MCF7 human breast cancer cells and HEK-293T [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % heat-inactivated fetal calf serum (Gibco) and grown at 37°C in a water-saturated 5% CO2 atmosphere. Transient transfections were performed by means of the PEI Exgen 500 procedure (Euromedex). Luciferase activity was determined 24 h after transfection and normalized with respect to the β-galactosidase activity (used as an internal control of transfection efficiency) as described previously [11]. Values are means ± S.E.M. for at least three independent experiments. SIRNA SMART pool (Dharmacon) was used to knock down CoAA in the cells. Interferin (Polysplus transfection) was used according to the manufacturer’s protocol to transfect cells with siRNA (small interfering RNA).

Immunoprecipitation and Western blot analyses

U2OS cells were lysed in co-immunoprecipitation buffer [50 mM Tris/HCl (pH 7.5), 125 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 0.5 % Triton X-100] with 1× Complete™ protease inhibitor, and the cell extract was centrifuged at 20,000 g for 30 min at 4°C. The supernatant was immunoprecipitated overnight with an anti-ERM antibody (anti-ERM[355-510]) [12,14,28,29] or pre-immune serum, and then incubated with Protein A–Sepharose beads for an additional 1 h at 4°C. After washing with lysis buffer, the immunopurified proteins were eluted with Laemmli sample buffer. Immunopurified proteins and total cell extracts were analysed by SDS/PAGE and then electrophoretically transferred to nitrocellulose membranes. Immunoblot analyses were performed with a rabbit anti-ERM antibody (anti-ERM[12-226]), which cross-reacts with both other PEA3 group members [30]), or anti-CoAA (Bethyl), anti-FLAG (Sigma), anti-Myc (Sigma) or anti-actin (sc1616, Santa Cruz Biotechnology) antibodies. This was followed by treatment with HRP (horseradish peroxidase)-conjugated secondary antibody or HRP-conjugated anti-rabbit IgG TrueBlot (eBioscience). Immune complexes were visualized by ECL (enhanced chemiluminescence) according to the manufacturer’s instructions (Santa Cruz Biotechnology). Prestained broad-range molecular mass markers (New England BioLabs) were used as standards in each SDS/PAGE.

RT (reverse transcription)–PCR

TRI Reagent (Molecular Research Center) was used as described by the manufacturer to isolate RNA from control or CoAA-down-regulated HeLa cells transfected with 200 ng of pSG5 ERM. The corresponding cDNAs were synthesized with the High Archive cDNA kit (Applied Biosystems). Quantitative PCR was performed with the brilliant SYBRgreen qPCR (quantitative PCR) master mix (Stratagene) in an MX3005P instrument as described by the supplier, with the following specific primers: GAPDH (glyceraldehyde-3-phosphate dehydrogenase)
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Figure 1 ERM and CoAA interact in vitro and in vivo

(A) Isolation of CoAA. HeLa cell nuclear extract was incubated for 2 h at 4°C with biotinylated ERM1-370 (biotin-tagged ERM) or biotinylated tag (control) purified on streptavidin-coupled beads. After extensive washing, the captured proteins were eluted in Laemmli buffer, separated by SDS/PAGE, stained with Coomassie Blue, and identified by MS. Band 1, CBP; band 2, CoAA.

(B) In-vitro-translated [35S]methionine-labelled CoAA was incubated with GST or GST–ERM. Input, 10% of the amount of in-vitro-translated [35S]methionine-labelled CoAA used in the reaction.

(C) Co-immunoprecipitation (IP) of endogenous ERM and CoAA from U2OS cells. Immunoprecipitation was performed with anti-ERM antibody or pre-immune serum (Pre) and proteins were detected as indicated with ERM- and CoAA-specific antibodies. The asterisk denotes a non-specific band.

(D) In-vitro-translated [35S]methionine-labelled ERM, PEA3 or ER81 was incubated with GST or GST–CoAA. Input, 10% of the amount of in-vitro-translated [35S]methionine-labelled protein used in the reaction.

In vitro migration assay

The in vitro migration assay was performed using a modified Boyden chamber complete with a membrane containing 8 μm pores in 24-well plates (BD Biosciences). MCF7 cells were transfected with plasmids using FuGENE® HD (Roche) and/or retransfected 6 h later with siRNA using INTERFERin™ (PolyPlus). After 24 h, cells were trypsinized and resuspended in culture medium. A total of 2×10⁵ or 5×10⁵ cells were added to the upper compartment of the transwell chamber. The lower chamber was filled with medium. At 24 h later, the cells were fixed with 4% paraformaldehyde and stained with 0.5% Crystal Violet. The cells remaining on the upper surface of the filter membrane were then completely removed by wiping with a cotton swab. For each well, the migrated cells were visualized and counted from three randomly selected fields under an inverted microscope. A migration assay was also performed in the presence of mitomycin C (3 μg/ml) to inhibit cell proliferation (Figures 6A and 6B).

RESULTS

ERM and CoAA interact in vitro and in vivo

In an attempt to isolate new nuclear proteins that might functionally modulate ERM transcriptional activity, we used a biotin-tagged ERM fragment (amino acids 1–370) or the biotinylated tag alone (control) immobilized on streptavidin beads as bait (Figure 1A). ERM-bound nuclear proteins were then identified by MS. Purification of ERM partners was performed four times. Among the ERM-bound nuclear proteins, we reproducibly identified CBP (band 1, Figure 1A), a known partner of the PEA3 group members [13,15,16], and ICAM1 (sense 5'-AGGGAGAGACACTGCAGACA-3' and antisense 5'-TGGCTTCGTCAGA TCACGTT-3').

(sense 5'-AAGGTGGTGAAGCAGGCGT-3' and antisense
5'-AGGTTCCACCCACCTGTG-3') and ICAM1.
As the three PEA3 group members present a high sequence homology, the in vitro study was extended to the other PEA3 group members, ER81 and PEA3. Like ERM, these transcription factors were found to interact strongly with GST–CoAA in GST pull-down experiments (Figure 1D). These results clearly identify CoAA as a binding partner for the PEA3 group members.

Mapping of the CoAA–ERM interaction domains

To narrow down the CoAA and ERM regions necessary for the interaction of these proteins, we performed GST pull-down experiments with different mutant and deletion constructs. We first used (Figure 2A, left-hand panel) the natural CoAA splice variant CoAM, containing only the two N-terminal RRM domains, and an N-terminal deletion mutant lacking these domains (CoAAC). CoAM was found to interact very poorly, and CoAAC strongly, with GST–ERM (Figure 2A, right-hand panel), suggesting that the C-terminal part of CoAA containing the YQ domain is mainly responsible for the ERM–CoAA interaction. We then tested the AxxQ mutant, where all the tyrosine residues of the YQ domain have been replaced with alanine residues [24]. This mutant was unable to bind GST–ERM, further indicating that the ERM–CoAA interaction depends on the YQ domain. Similar results were observed for PEA3 and ER81 (results not shown).

We next mapped the CoAA-interacting domain of ERM. ERM shares a similar architecture with the two other PEA3 group members: an N-terminal transactivation domain and the ETS DNA-binding domain located on the extremity of the C-terminus (Figure 2B, left-hand panel). As shown in Figure 2(B), CoAA interacted strongly with ERM1–510 and ERM1–370, but not with ERM354–510, indicating that the ETS DNA-binding domain and the C-terminal part of ERM are totally dispensable for the ERM–CoAA interaction. Further ERM C-terminal deletions progressively decreased the CoAA interaction (Figure 2C, compare ERM 1–370, ERM1–298 and ERM1–226), indicating that residues 226–370 of ERM are important for CoAA recruitment. Similar results were obtained with progressive ERM N-terminal deletion constructs (Figure 2C, compare ERM1–510, ERM1–298 and ERM1–226), indicating that residues 226–370 of ERM are important for CoAA recruitment. Taken together, these results suggest that the ERM region between residues 112 and 298 is necessary for the optimal interaction with the YQ domain of CoAA.

CoAA overexpression increases the PEA3 group member transcriptional activity

We next assessed the effect of CoAA on PEA3 group member transcriptional activity. Luciferase reporter gene assays were first performed with ERM and the EndoA–Luc reporter plasmid. As
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Figure 3 CoAA increases the PEA3 group member transcriptional activity

(A) Left-hand panel: RK13 cells were transfected without (Ctrl) or with ERM and an increasing quantity of CoAA (from 2.5 to 20 ng of FLAG–CoAA plasmid) together with the EndoA–Luc reporter plasmid. *P < 0.01. Right-hand panel: RK13 cells were transfected with vectors expressing ERM and CoAA or AxxQ, and protein levels were assessed by immunoblotting as indicated. (B) CoAA increases ERM-dependent transcription of the ICAM1 promoter. RK13 cells were transfected with the ICAM1–Luc reporter plasmid and with plasmids bearing the ERM and CoAA or AxxQ genes, *P < 0.01. (C) U2OS cells were transfected without (Ctrl) or with E2F1 and an increasing quantity of CoAA (from 20 to 200 ng of FLAG–CoAA plasmid) together with the 5xE2F–Luc reporter plasmid. (D) RK13 cells were transfected with ERM, ER81 or PEA3 with CoAA or AxxQ together with the EndoA–Luc reporter, *P < 0.01.

CoAA RRMs are required for CoAA-mediated activation of ERM activity

As the RRMs of CoAA are not required for the CoAA–ERM interaction, we next investigated whether they are necessary for CoAA-dependent activation of ERM. Luciferase reporter gene assays were performed with CoAAC (CoAA without its RRMs). In contrast with CoAA, this deletion mutant was unable to stimulate ERM-induced transcription of the EndoA–Luc reporter construct (Figure 4A), although its level of production was similar (Figure 4A) and it still interacted with ERM (Figure 4B). This suggests that the interaction with ERM is not sufficient to induce transcriptional activation and that the RRMs are required in this process. The RRM domains share a conserved structure containing two short conserved sequences called RNP-1 and RNP-2 (ribonucleoprotein domains 1 and 2), forming a four-stranded β-sheet backed by two α-helices [35]. Interestingly, CoAA shares a similar architecture with two other hnRNP-related proteins, p54nrb and hnRNPA1: two N-terminal RRMs followed by an auxiliary domain. Furthermore, the RRMs of both hnRNPA1 and p54nrb can partially replace the RRMs of CoAA in mediating some of the transcriptional effects of CoAA [22]. We thus evaluated the effect of the RRMs of CoAA on ERM by replacing them with the related RRMs of hnRNPA1. As shown in Figure 4(B), A1N-CoAAC (CoAA with the RRMs of hnRNPA1), in contrast with hnRNPA1, was found to interact with ERM,
confirming that the YQ domain is sufficient to recruit ERM. Like hnRNPA1, however, A1N-CoAAC was unable to stimulate ERM-induced transcription of the EndoA reporter construct (Figure 4A). Similar results were obtained with the N-terminal RRM of p54nrb (results not shown). Taken together, our results indicate that the RRMs of CoAA are required to mediate the effect of CoAA on ERM transcriptional activity, even though they are not required for the CoAA–ERM interaction.

**Knockdown of CoAA decreases ERM transcriptional activity**

We then carried out RNA interference experiments to determine the effect of knockdown of CoAA on ERM transcriptional activity. At 2 days after transfection of HeLa cells with the CoAA siRNA, the CoAA protein level was clearly diminished (Figure 5A). We then used qPCR to estimate, in HeLa cells overexpressing ERM, the effect of CoAA knockdown on transcription of the endogenous *ICAM1* gene (an ERM target gene [28]) (Figure 5B). In the absence of ERM, the *ICAM1* mRNA level was found to increase slightly, but significantly, in response to knockdown of endogenous CoAA. As predicted, a major increase in this level was observed when ERM was expressed. CoAA knockdown, however, was found to reduce the level of *ICAM1* mRNA observed in the presence of overexpressed ERM (Figure 5B). Similarly, siRNA knockdown of endogenous CoAA causes a decrease in PEA3-dependent transcription of the *ICAM1* promoter (results not shown). This suggests that endogenous CoAA is involved in the regulation of the PEA3 group member transcriptional activity.
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CoAA participates in the PEA3 migratory activity on MCF7 human cancer cells

Since PEA3 has been reported to stimulate the migration of MCF7 cells [4], we examined whether CoAA could be implicated in this process. At 1 day after transfection of MCF7 cells with CoAA and/or PEA3 plasmid, CoAA- and PEA3-triggered migration was examined using the transwell assay in the presence of mitomycin C to eliminate the contribution of cell proliferation. As expected, PEA3 overexpression induced significant motility of MCF7 cells (Figures 6A and 6B). Interestingly, cell migration in the presence of both CoAA and PEA3 was more than the sum of the effects of CoAA or PEA3 alone, suggesting a synergistic effect. This synergy was not observed between PEA3 and AxxQ (results not shown).

To assess the effect of the depletion of CoAA on the PEA3-induced migratory activity, we carried out similar experiments by transfecting MCF7 cells with siRNA directed against CoAA (siRNA CoAA, Figures 6C and 6D). When combined with PEA3 overexpression, siRNA CoAA appeared to counteract the migration-enhancing action of PEA3. Taken together, these results suggest that CoAA is important for the effects of PEA3 on cell migration.

DISCUSSION

In the present study we have identified CoAA as a new ERM partner and extended the in vitro interaction to the two other PEA3 group members PEA3 and ER81. Moreover we show that ERM interacts with CoAA in living cells and that the CoAA domain involved in this interaction is the YQ domain. Five CoAA interactors have been identified previously: the coactivator TRBP and the histone acetyltransferase CBP [17], the proto-oncogene coactivator SYT [18,19], the extracellular domain of the GH (growth hormone) receptor called GHBP (GH-binding protein) [36] and the transcription factor RUNX2 [20]. Although the domain of CoAA involved in the interaction with GHBP and CBP remains to be determined, the other three partners are known to require the YQ domain [17,18,20]. This domain contains more than 20 XYXXQ motifs (X denotes a small amino acid residue, i.e. glycine, alanine, serine or proline) [17] and exhibits transcriptional activity [18]. Previous studies have shown that the aromatic residue repeats interspersed among the small residues are important for allowing protein–protein interactions [17,19,37,38]. The present study confirms that this motif plays a crucial role in such interactions and particularly highlights the central role of the tyrosine residues, since their replacement with alanine residues abolishes the...
interaction with the PEA3 group members. The YQ domain is also present in the EWS, TLS/FUS and SYT [18,24] oncoproteins, and it would be interesting to test whether it allows the interaction of these proteins with PEA3 group members, as seen with RUNX2, which interacts with CoAA, TLS/FUS and EWS [39].

The interaction domains defined in the known CoAA partners are relatively large regions: the SYT C-terminal transactivating domain (QPQG domain, amino acids 73–387) [18], the C-terminus (amino acids 1641–2063) and LXXLL-containing region (amino acids 719–999) of TRBP [17], the DNA-binding domain of RUNX2 (amino acids 50–179) [20], and the C-terminal region (amino acids 1195–2414) of p300/CBP [17]. Remarkably, no sequence homology is observed between these domains. The integrity of the ERM central region (amino acids 112–298) appears necessary for an optimal interaction with CoAA. This region shows no significant homology with the interaction domains of the other CoAA partners. Interestingly, as observed with the SYT QPQG domain [18] and the TRBP domain [17], the 112–298 region of all three PEA3 group members are proline-rich (proline content of approximately 15%). As previous studies have shown that proline-rich regions are important for protein–protein interactions [40,41], a proline-rich region might be one of the features necessary for CoAA binding to interacting domains.

We further show that CoAA increases the transcription-enhancing action of PEA3 group members of the EndoA and ICAM1 promoters in RK13 cells. This activating function was also observed in human cells (such as HeLa and U2OS cells, results not shown). Although CoAA can up-regulate the basal transcription of MMTV (murine mammary tumour virus)– and CMV–luciferase reporter genes in HeLa cells [22], it appears not to significantly increase basal EndoA- or ICAM1-promoter-driven transcription. Consistent with its role as a coactivator, siRNA knockdown of endogenous CoAA causes a decrease in PEA3 group member-dependent transcription. CoAA siRNA was also found to increase the basal expression of ICAM-1 in the absence of PEA3 group member expression, suggesting that CoAA may regulate the expression of ICAM-1 in HeLa cells via an additional mechanism that does not require CoAA–PEA3 group members binding.

To date, the activating function of CoAA has been observed with the coactivators SYT, CBP, TRBP and GHBP [17–19,36]. On the other hand, the CoAA interaction with the DNA-binding domain of RUNX2 has been found to decrease RUNX2 binding to its target gene [20]. As RUNX2 can act as a repressor or an activator, its interaction with CoAA can increase or decrease the expression of its target genes. However, CoAA does not interact with the ERM DNA-binding domain. Furthermore, we have observed enhancement by CoAA of the transcriptional activity of an ERM construct in which the ETS domain was replaced with the Gal4 DNA-binding domain (results not shown). This strongly suggests that CoAA-dependent PEA3 group member activation does not depend on the DNA-binding process. Moreover, CoAA stimulates PEA3 group member transcriptional activity without affecting significantly their level or cellular localization (results not shown). It thus likely modulates PEA3 group member transcriptional activity by recruiting coactivators, preventing interaction with corepressors, or modulating PEA3 group member post-translational modifications. Further work will be required to determine which of these mechanisms are involved.

CoAA is an hnRNP-like protein with two N-terminal RRM.s. It is involved in promoter-specific mRNA splicing as well as transcriptional activation [21–23]. As the luciferase reporter constructs used in our experiments have no introns, the experiments carried out in the present study could tell us nothing about CoAA splicing effects. As deletion or substitution of the CoAA RRM domains can completely abolish the stimulation of ERM transcriptional activity by CoAA without affecting the latters binding to ERM, direct interaction between CoAA and ERM appears insufficient to promote the ERM-activating effect of CoAA. Furthermore, our results emphasize the importance and specificity of the CoAA N-terminal RRMs in this process.

Interestingly, similar deletion of the CoAA RRMs affects the response of MMTV reporter constructs to dexamethasone treatment [17]. Moreover, Auboeuf et al. [22] have demonstrated that the RRMs of CoAA are necessary for transcription from CMV reporter constructs. The functional role of the RRMs in CoAA-mediated enhancement of ERM transcriptional activity leads us to suppose that they are necessary for contact with coactivators or regulatory complexes. These coactivators are probably neither SYT nor TRBP, since they bind directly to the YQ domain. In support of this hypothesis, CoAAC (which contains the YQ domain and can still interact with ERM) causes dose-dependent inhibition of ERM transcriptional activity (results not shown), probably by competing with endogenous CoAA.

In terms of functional effect, the results of the present study demonstrate that the co-expression of CoAA and PEA3 triggers an important increase of MCF7 migration and that CoAA knockdown counteracts the PEA3-induced migratory activity. The PEA3 group members are well known to act as positive regulators of the expression of genes associated with proliferation, transformation, migration and invasion [2]. The enhancement of the migratory effect of PEA3 mediated by CoAA must probably be correlated with the increase of PEA3 target genes. Taken together, the results of the present study suggest that CoAA may be an important regulator of PEA3 group member activity during metastasis.

**AUTHOR CONTRIBUTION**

Kathye Verreman, Jean-Luc Baert, Alexis Verger, Hervé Drobecq, Elisabeth Ferreira and Didier Monte performed the experiments; Kathye Verreman, Jean-Luc Baert, Alexis Verger, Yvan de Launoit and Didier Monte discussed and interpreted all of the results; Kathye Verreman wrote the paper in collaboration with Alexis Verger, Jean-Luc Baert, Yvan de Launoit and Didier Monte.

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