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# The Receptor-associated Coactivator 3 Activates Transcription through CREB-binding Protein Recruitment and Autoregulation\*

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**Transcriptional coactivators are involved in gene activation by nuclear hormone receptors. The receptor-associated coactivator 3 (RAC3) was recently identified to be highly related to the steroid receptor coactivator-1 and transcriptional intermediate factor 2, thereby establishing a novel family of nuclear receptor coactivators. In this study, we identified a RAC3 fragment containing three LXXLL motifs conserved among this family, which is sufficient to mediate nuclear receptor interaction *in vivo* and *in vitro*. Point mutations that disrupt ligand-dependent activation function of the receptor inhibited the interaction. We found that a 162-amino acid fragment of RAC3 conferred transcriptional activation and recruited the CREB-binding protein and that three distinct LXXLL motifs mediated the transcriptional activation. A trimeric far Western analysis demonstrated the formation of a ternary complex containing CREB-binding protein, RAC3, and the receptor. In addition, we showed that RAC3, transcriptional intermediate factor 2, and steroid receptor coactivator-1 are expressed in specific tissues and cancer cells and that RAC3 transcript is directly up-regulated by retinoid treatment. These results suggest that RAC3 may contribute to amplified transcriptional responses through both recruitment of additional coactivators and autoregulation by the receptor-coactivator complex.**

Transcriptional regulation by nuclear receptors for steroids, thyroid hormones (TR),<sup>1</sup> retinoids (RAR), and vitamin D<sub>3</sub> controls key aspects of animal development, reproduction, homeostasis, and adult organ physiology (for reviews, see Refs. 1–5). The nuclear receptors are characterized by a common domain structure, including an N-terminal A/B region that contains the first activation function (AF-1), a DNA binding domain (DBD) responsible for recognition of specific DNA response elements, and a C-terminal ligand binding domain. The ligand binding domains of TR and RAR also mediate heterodimeriza-

tion with retinoid X receptor (RXR) (for review, see Ref. 2), and such complexes are capable of repressing basal transcription in the absence of ligand, while activating net transcription upon ligand treatment (6). Although the mechanisms of such repression and activation functions are not fully understood, a growing list of cofactors that interact with and modulate the transcriptional activation and repression properties of nuclear receptors have been identified (for reviews, see Refs. 7–9).

The involvement of cofactors in nuclear receptor signaling was first postulated when members of the nuclear receptor superfamily were found to cross-react with each other functionally (10) and with other transcription factors (11). Currently, several such cofactors have been identified biochemically (12–16) and genetically (17, 18). In particular, two related proteins known as the silencing mediator for RAR and TR (SMRT) and the nuclear receptor corepressor (N-COR) were identified as TR- and RAR-interacting proteins that can assist transcriptional repression by the unliganded receptors (17–21). Recently, SMRT and N-COR were found to form complexes with the transcriptional corepressor mSin3 and the histone deacetylases HDAC1 and mRPD3 (22–24), suggesting that transcriptional repression by SMRT and N-COR might involve histone deacetylation (25, 26).

Hormone binding is thought to dissociate the corepressor complex from nuclear receptors, allowing the receptors to recruit coactivators, such as the CREB/E1A-binding proteins (CBP/p300) (for review, see Ref. 27 and references therein), the p300/CBP-associated factor (P/CAF; Ref. 28), and the steroid/nuclear receptor coactivator (SRC) family proteins (for review, see Ref. 9). In contrast to the nuclear receptor corepressor complexes, all of these putative coactivators contain intrinsic histone acetyltransferase (HAT) activity (29–31). Therefore, transcriptional activation and repression by nuclear receptors might be determined by the relative acetylation level of targets common to nuclear receptor coactivators and corepressors. Recent findings also suggest that, in addition to histones, these coactivators may also acetylate and modulate the DNA binding property of activator proteins or general transcription factors (32, 33), suggesting the involvement of multiple targets and pathways in transcriptional regulation.

The steroid/nuclear receptor coactivator gene family contains the steroid receptor coactivator-1 (*SRC-1*; also known as *NCoA-1*) (34–38), the transcriptional intermediate factor 2 (*TIF2*, also known as *GRIP1*) (39–41), and the receptor-associated coactivator 3 (*RAC3*, also known as *ACTR*, *p/CIP*, and *AIB1*) (42–44). Sequence comparison of these proteins reveals that they share an overall identity of about 40%, but with striking similarity at the N-terminal basic helix-loop-helix (bHLH) and period-aryl hydrocarbon receptor-single minded (PAS) “A” and “B” domains. The bHLH-PAS domain function in protein-protein interactions, heterodimerization, and target gene selection in many members of this family (45–47), but the

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<sup>1</sup> The abbreviations used are: TR, thyroid hormone receptor; RAC3, receptor-associated coactivator 3; SRC, steroid receptor coactivator; AF, activation function; AF-2, ligand-dependent AF; AF2-AD, AF-2 activation domain; DBD, DNA binding domain; CBP, CREB-binding protein; RAR, retinoid receptor; HAT, histone acetyltransferase; LCD, leucine-charged domain; RID, receptor-interacting domain; Gal, galactosidase; poly-Q, polyglutamine; bHLH, basic helix-loop-helix; PAS, period-aryl hydrocarbon receptor-single-minded.

role of this domain in the SRC family remains unclear. In addition to the bHLH-PAS domain, seven highly conserved motifs containing a LXXLL (L indicates hydrophobic residue) core consensus sequence flanked by charged and conserved residues are found in a central region that mediates both nuclear receptor interaction and transcriptional activation functions of all three SRC proteins (42, 43, 48). These motifs are also known as the leucine-charged domains (LCDs). Three of these motifs in SRC-1 have been shown to mediate direct protein-protein interaction with liganded receptors (43, 48), suggesting that these conserved motifs might play an important role in the functions of these coactivators.

In this study, we further characterized the nuclear receptor interaction and transcriptional activation domains of RAC3 and found that different LXXLL motifs are differentially involved in either nuclear receptor interaction or transcriptional activation, suggesting the importance of the flanking sequences in determining the function of these LXXLL motifs. We showed that RAC3 interacts directly with CBP through its activation domain and that RAC3 can form a bridge for the interaction between CBP and nuclear receptors, suggesting that one mechanism of transcriptional activation by these coactivators is recruitment of additional cofactors. In addition, we found that the RAC3 transcript is directly up-regulated by retinoic acid, demonstrating a new and perhaps independent mechanism of transcriptional coactivation by the nuclear receptor coactivators. Therefore, these results strongly suggest that RAC3 plays an important role in nuclear receptor activation through utilization of multiple mechanisms.

#### MATERIALS AND METHODS

**Plasmids**—The individual RAC3 fragments were obtained by either restriction enzyme digestion of the full-length RAC3 clone or by PCR amplification with synthetic primers harboring suitable cloning sites. The LXXLL motif constructs were generated by subcloning annealed double-stranded oligonucleotides encoding the specified amino acids into the yeast expression vector pAS1 (49). Detailed information regarding these constructs is available at <http://146.189.26.74/>. All other constructs have been previously published (18, 28, 39, 42, 50, 51).

**Far Western Analysis**—GST fusion proteins were overexpressed and purified from DH5 $\alpha$  cells on glutathione-agarose beads. The fusion proteins were separated on denaturing protein gels (SDS-polyacrylamide gel electrophoresis) and electroblotted onto nitrocellulose filters in a transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.01% SDS). After denaturation in 6 M guanidine hydrochloride, the proteins were renatured by stepwise dilution of guanidine hydrochloride to 0.187 M in HB buffer (25 mM Hepes, pH 7.7, 25 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol). The filters were then saturated with blocking buffer (5% nonfat milk and then 1% milk in HB buffer plus 0.05% Nonidet P-40). *In vitro* translated <sup>35</sup>S-labeled proteins were generated in reticulocyte lysate (Promega) and were diluted into a hybridization buffer (20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 0.05% Nonidet P-40, 1% milk, 1 mM dithiothreitol). The filters were allowed to hybridize overnight at 4 °C followed by three washes with hybridization buffer. The bound <sup>35</sup>S-labeled proteins were detected by autoradiography.

**Yeast Two-hybrid Assay**—The yeast two-hybrid assay was carried out in the yeast Y190 strain (49). The GAL4 DBD fusion constructs were generated in the pGBT9 vector, and the GAL4 activation domain (AD) fusion constructs were made in the pGAD424 or pACTII vector (CLONTECH). The  $\beta$ -galactosidase activities were determined with the ONPG liquid assay as described previously (52).

**Cell Culture and Transient Transfection**—African green monkey kidney CV-1 cells were grown at 37 °C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium supplemented with 10% resin-charcoal stripped fetal bovine serum, 50 units/ml penicillin G, and 50  $\mu$ g/ml streptomycin sulfate. One day prior to transfection, cells were plated in 24-well culture dishes at a density of 50,000 cells/well. Transfection was performed by standard calcium phosphate precipitation. All transfection experiments were performed in triplicate and were replicated at least once. Twelve hours after transfection, cells were washed with PBS and refed fresh medium containing the indicated amounts of ligand. After 30 h, cells were harvested and  $\beta$ -galactosidase and luciferase activities

were assayed as described previously (52).

**Luciferase and  $\beta$ -Galactosidase Assay**—Transfected cells in each well were lysed as described (52) and processed for the luciferase and the  $\beta$ -galactosidase assays. The lysates were transferred into 96-well microtiter plates for luciferase assay and 96-well microtiter plates for  $\beta$ -galactosidase assay as described (52). The luciferase activities were determined with a MLX microtiter plate luminometer (Dynex) using 100  $\mu$ l of assay solution (0.1 M KPO<sub>4</sub>, 5 mM ATP, 10 mM MgCl<sub>2</sub>) and 100  $\mu$ l of luciferin solution (0.01 M D-luciferin in 0.1 M KPO<sub>4</sub>, pH 7.8). The luciferase activities were normalized to the  $\beta$ -galactosidase activity expressed from the cotransfected pCMX- $\beta$ Gal plasmid.

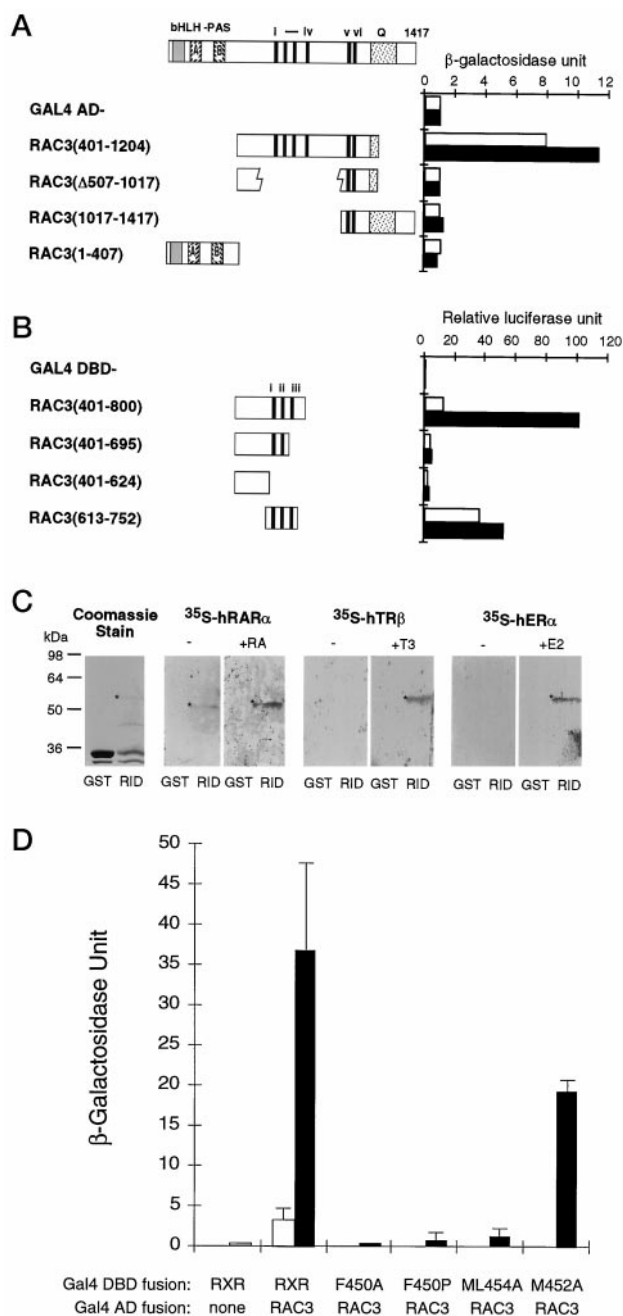
**Northern Blot Analysis**—Cells were treated with different concentrations of atRA (0, 10, 100, or 1000 nM) for a period of 12 h. In the control group, cells were treated with an equal volume of solvent (80% EtOH plus 20% DMSO). Total RNAs were isolated using an RNA isolation kit (RNAzol) and separated on a 1% agarose gel with 2.2 M formaldehyde and blotted onto nitrocellulose filters. Filters were hybridized with random-primed <sup>32</sup>P-labeled DNA probes specific for RAC3, TIF2, and SRC-1 in hybridization buffer (50% formamide, 5 $\times$  SSPE (1 $\times$  SSPE is 0.18 M NaCl, 10 mM NaPO<sub>4</sub>, and 1 mM EDTA at pH 7.7), 2 $\times$  Denhardt's solution, 0.1% SDS, 0.1 mg of sheared herring sperm DNA/ml) at 42 °C overnight. The final wash was in 1 $\times$  SSC (0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at 65 °C. Filters were exposed to x-ray film at -70 °C for about 24 h. The human tissue and cancer cell blots were purchased from CLONTECH and hybridized according to manufacturer's protocols.

#### RESULTS

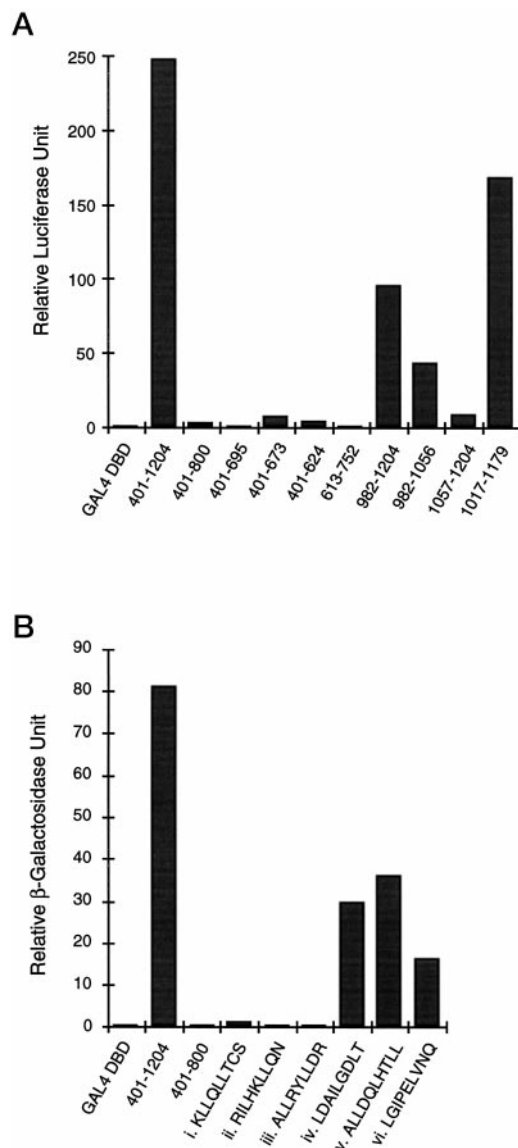
**RAC3-Nuclear Receptor Interaction**—The nuclear receptor-interacting domain of RAC3 has been previously located within a central fragment between amino acids 401 and 1204 (42). Here, we show that deletion of amino acids 507–1017 eliminates the receptor interaction and that the rest of the N-terminal and C-terminal sequences cannot interact with liganded RAR (Fig. 1A) or other class II receptors (data not shown) in the yeast two-hybrid system, suggesting that amino acids 507–1017 contain the interacting domain. The interaction was then confirmed by the mammalian two-hybrid system, in which amino acid fragments 401–800 and 613–752 both interact with RAR, whereas fragments 401–695 and 401–624 do not interact with the receptor (Fig. 1B). We found that the 613–752 fragment can also interact significantly with the unliganded receptor, and such interaction was only slightly enhanced by ligand, whereas the interaction with fragment 401–800 is ligand-dependent. Coincident with previous studies (43, 48), this receptor-interacting domain (RID) of RAC3 contains three LXXLL motifs, which have been implicated in mediating direct interaction between SRC-1 and nuclear receptors (43, 48). To further confirm the interactions between RAC3-RID and nuclear receptors, we generated a GST-RID fusion and analyzed its interaction with three nuclear receptors *in vitro* by far Western blot analyses. Significant interactions were observed with liganded RAR, TR, and estrogen receptor (Fig. 1C), demonstrating that this RID domain indeed mediates a ligand-dependent interaction between RAC3 and nuclear receptors.

**Point Mutations of the AF2-AD That Abolish RAC3 Interaction**—We have demonstrated previously that the interaction between RAC3 and nuclear receptor depends on the presence of an intact AF2-AD (42). Here, we further tested whether the AF-2 function *per se* is required for such interaction. Several point mutations within the AF2-AD helix of the human RXR $\alpha$  (51) were analyzed for their effects on interaction with RAC3. We found that disruption of the AF-2 function by three independent AF2-AD point mutations (F450A, F450P, and ML454A) abolishes the interaction between RAC3 and RXR (Fig. 1D). In contrast, a neutral AF2-AD mutation (M452A) permits a strong ligand-dependent interaction. These results suggest that the activation function of the AF2-AD helix correlates with its ability to interact with RAC3, further supporting the hypothesis that RAC3 is an AF-2-dependent nuclear receptor coactivator.





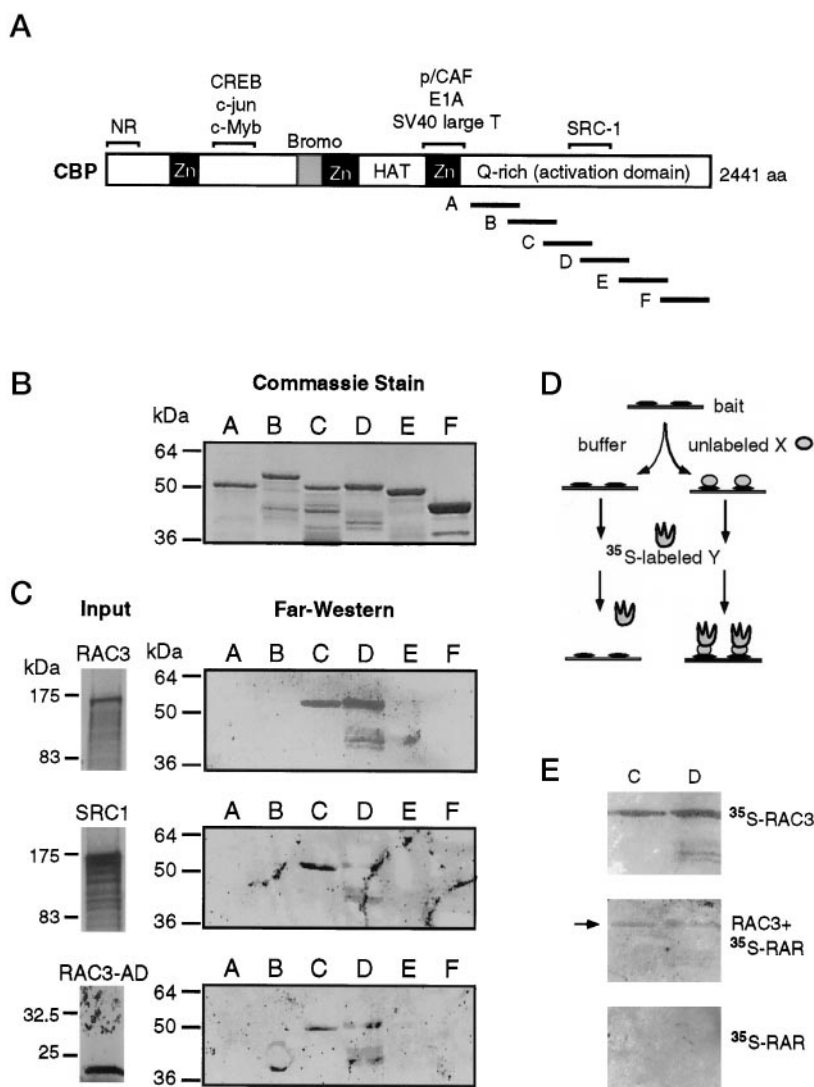
**FIG. 1. RAC3-nuclear receptor interaction.** *A*, the indicated RAC3 fragments, with starting and ending amino acids shown in the parentheses, are expressed as GAL4 AD fusions in the yeast pGAD424 vector. Individual pGAD-RAC3 constructs were cotransformed with a pGBT-F.RAR construct, which expresses a GAL4 DBD-full-length hRAR $\alpha$  fusion. Relative  $\beta$ -galactosidase activities were determined from three independent transformants in the absence (*open bars*) or presence (*closed bars*) of 1  $\mu$ M atRA. The corresponding RAC3 domains are shown at the *top* as described previously (42). *bHLH-PAS*, basic helix-loop-helix-Per/Ahr/Sim domain; *i-vi*, the LXXLL motifs; *Q*, the polyglutamine tract. *B*, the indicated RAC3 fragments were expressed as GAL4 DBD fusions in the pCMX-Gal vector (52) and cotransfected together with a VP16-RAR fusion construct that expresses a VP16 AD-full-length hRAR $\alpha$  fusion. Relative luciferase activities were determined from three independent transfections in the absence (*open bars*) or presence (*closed bars*) of 1  $\mu$ M atRA. *i*, *ii*, and *iii* indicate the three LXXLL motifs. *C*, interaction of RAC3 with nuclear receptors *in vitro*. The purified GST module (*GST*) and the GST-RAC3-RID (*RID*) fusion were separated by SDS-polyacrylamide gel electrophoresis and analyzed by far Western analyses for interaction with  $^{35}$ S-labeled hRAR $\alpha$ , hTR $\beta$ , and hER $\alpha$  in the absence (–) or presence (+) of 1  $\mu$ M cognate ligands. The positions of the intact GST-RAC3-RID fusion proteins are marked with *asterisks*. *T3*, 3,5,3'-triiodo-L-thyronine; *E2*, 17 $\beta$ -estradiol; *RA*, *all-trans* retinoic acid. *D*, receptor AF2-AD point



**FIG. 2. The RAC3 activation domain.** *A*, transcriptional activation by RAC3 in mammalian cells. The indicated RAC3 fragments were expressed as GAL4 DBD fusions in the pCMX-Gal vector. The relative fold induction was determined by comparison with the pCMX-Gal vector. *B*, three individual LCD motifs activate transcription. The indicated RAC3 fragments or individual motifs were expressed as GAL4 DBD fusions in the yeast Y190 cells, and the  $\beta$ -galactosidase units were determined from three independent transformants.

*Three Conserved LXXLL Motifs Activate Transcription*—The transcriptional AD of RAC3 was previously located within amino acids 401–1204 (42). Further deletional analyses in yeast cells reveal that the AD of RAC3 is located at the C terminus of this central fragment (Fig. 2A), in contrast to the receptor-interacting domain found at the N terminus. In particular, amino acids 401–800 and further deletion mutants cannot activate transcription from a Gal4-dependent  $\beta$ -galactosidase reporter, whereas the C-terminal fragments 982–1204, 982–1056, and 1017–1179 strongly stimulate the reporter gene expression (Fig. 2A). These results demonstrate a

mutations disrupt RAC3 interaction. The interactions between RAC3 and AF2-AD point mutants were determined in the yeast two-hybrid system. The RAC3.1 fragment was expressed as a GAL4 AD fusion, whereas RXR mutants were expressed as GAL4 DBD fusions. The  $\beta$ -galactosidase activities were determined from three transformants in the absence (*open bars*) or presence (*closed bars*) of 1  $\mu$ M 9-*cis*-RA.



**FIG. 3. RAC3 interacts with CBP and forms a ternary complex with CBP and RAR.** *A*, schematic diagram of the CBP domain structure and CBP fragments (*A–F*) (28). The various factors that interact with each CBP domain are shown at the *top*. *NR*, nuclear receptors; *Bromo*, bromodomain; *Zn*, zinc finger domain; *Q*, glutamine. *B*, Coomassie Blue staining of the purified GST-CBP fusion proteins. *Lanes A–F* correspond to the CBP fragments *A–F* shown in *panel A*. *C*, interaction of RAC3, RAC3-AD, and SRC-1 with CBP fragments in far Western analyses. The input probes used in each experiment are shown at the *left*. *D*, schematic diagram of the trimeric far Western protocol. *E*, RAC3 forms a complex with CBP and RAR simultaneously in the trimeric far Western blot. The *arrow* indicates the CBP-RAC3-RAR ternary complex.

minimal RAC3-AD between amino acids 1017 and 1179. Within the RAC3-AD, we found several conserved LXXLL motifs. Because these motifs are not required for nuclear receptor interaction, we speculate that they might be directly involved in transcriptional activation. We analyzed the activation function of each of these motifs and found that when fused with the GAL4 DBD, the three C-terminal motifs (iv, v, and vi), but not the N-terminal ones, can activate transcription from a Gal4-dependent luciferase reporter in yeast cells (Fig. 2*B*). These results indicate that these C-terminal LXXLL motifs may directly mediate transcriptional activation by RAC3.

**RAC3 Interacts with CBP and Nuclear Receptors Simultaneously**—Because SRC-1 can interact with the general transcriptional coactivator CBP and p300 (37), we tested whether RAC3 can also interact with CBP. Serial GST-CBP fusions covering the SRC-1-interacting domain were analyzed for interactions with <sup>35</sup>S-labeled full-length RAC3, SRC-1, and RAC3-AD (amino acids 1017–1179) (Fig. 3, *A–C*). We find that RAC3 interacts strongly with the CBP fragments C and D but not with other fragments. Similarly, SRC-1 interacts with CBP fragments C and D, consistent with previous observations (36, 37). We also find that the RAC3-AD alone is sufficient for CBP interaction, suggesting that one function of AD is to recruit CBP or its related proteins.

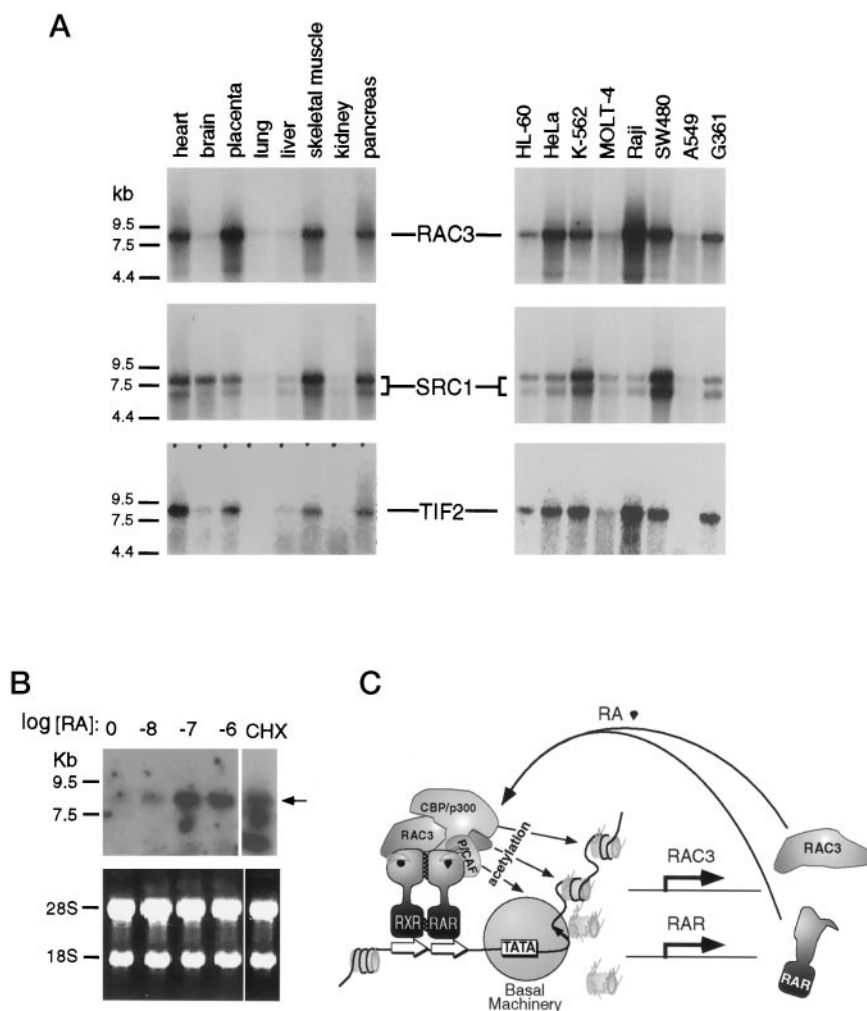
The ability of RAC3 to interact with both CBP and nuclear receptors prompted us to test whether each pair of interactions is enough to mediate the formation of a ternary complex. We

developed a trimeric far Western protocol to test this possibility (Fig. 3*D*). Briefly, the CBP fragments C and D were probed first with unlabeled full-length RAC3 and then with <sup>35</sup>S-labeled nuclear receptor. The results demonstrate that RAC3 can bridge the interaction between CBP and RAR in the presence of ligand (Fig. 3*E*) but not in the absence of ligand (not shown). As controls, CBP fragments C and D were probed parallel with <sup>35</sup>S-RAC3 and <sup>35</sup>S-RAR, individually. These results suggest that the strength of interactions between CBP-RAC3 and RAC3-RAR are sufficient to link together a ternary complex. These results also confirm that RAC3 utilizes distinct domains for interaction with CBP and RAR.

**RAC3 Is Expressed in a Tissue- and Cancer Cell-specific Manner**—The expression patterns of RAC3 in different human tissues and cancer cells were examined and compared with those of SRC-1 and TIF2. RAC3 is expressed at high levels in the heart, placenta, skeletal muscle, and pancreas but at very low levels in the brain, lung, liver, and kidney (Fig. 4*A*, *left*). The SRC-1 message is expressed at high levels in the heart, brain, placenta, skeletal muscle, and pancreas and, again, at extremely low levels in the lung, liver, and kidney. Two distinct SRC-1 messages are clearly detectable, and the larger message appears to be expressed at a higher level than the smaller form. The expression pattern of TIF2 is very similar to that of RAC3, but with the highest expression in the heart instead of placenta. In the human cancer cells, we found the highest expression of the RAC3 message in the Burkitt's lymphoma Raji cells

FIG. 4. Expression patterns of RAC3, SRC-1, and TIF2 in human tissues and cancer cells.

**A**, human multiple tissue (left) and cancer cell (right) Northern blots were first hybridized with a  $^{32}\text{P}$ -RAC3 DNA probe and then rehybridized with  $^{32}\text{P}$ -SRC-1 and then with  $^{32}\text{P}$ -TIF2 probes. Between each rehybridization, the membranes were exposed to x-ray film to ensure appropriate stripping of the earlier probe. Note that SRC-1 transcripts show different patterns from that of RAC3 and TIF2, indicating the absence of cross-contamination from each hybridization. **B**, total RNA (20  $\mu\text{g}$ ) of HL-60 cells treated with indicated concentrations of atRA for 12 h or with 1  $\mu\text{M}$  tRA plus 10  $\mu\text{M}$  cycloheximide (CHX) were analyzed for RAC3 message by Northern blot analysis (top panel). The position of the RAC3 transcript is indicated by an arrow. Ethidium bromide staining of the gel confirms that each lane contains an equal amount of RNA (bottom panel). **C**, model of the receptor-coactivator autoregulation. After ligand binding, the RXR-RAR heterodimer recruits a coactivator complex that contains members of the SRC family proteins CBP/p300 and p300/CBP-associated factor. This coactivator complex functions as an acetylator machinery that acetylates histones and disrupts nucleosome structure, allowing the access of basal transcriptional machinery to the core promoter. Because both RAR and RAC3 transcripts are elevated by RA treatment, the increased concentration of the two key proteins should further amplify the transcriptional responses, leading to a high level of gene induction.



and substantial expression in the epitheloid carcinoma HeLa cells, the chronic myelogenous leukemia K-562 cells, the colorectal adenocarcinoma SW480 cells, and the melanoma G361 cells. Low levels of RAC3 were found in promyelocytic leukemia HL-60 cells, the lymphoblastic leukemia MOLT-4 cells, and the lung carcinoma A549 cells. These results indicate that RAC3 expression varies greatly in different tissues and cancer cells. In contrast, the SRC-1 transcript is highly expressed only in K-562 cells and SW480 cells and not in the Raji cells. The expression pattern of TIF2 is very similar to that of RAC3, with the highest expression level in the Raji cells. Together, these results suggest that the expression of the SRC family genes is highly variable in different tissues and cell types, suggesting that members of this coactivator family might have distinct roles in different cell types.

**RAC3 Expression Is Up-regulated by Retinoic Acid**—The expression of nuclear receptor genes is frequently regulated by cognate hormones and, in particular, autoregulation of the RAR gene expression by atRA has been clearly demonstrated (53). Because RAC3 is involved in transcriptional activation of the receptor, we speculated that the expression of coactivator might be also under regulation by the ligand-receptor-coactivator complex. We analyzed the effect of atRA on RAC3 gene expression in the HL-60 cells and found that atRA significantly enhanced the expression of RAC3 in a concentration-dependent manner (Fig. 4B). Induction of the RAC3 transcript was most obvious at a concentration between  $10^{-8}$  and  $10^{-7}$  M of atRA and was not sensitive to inhibition of *de novo* protein synthesis. Similar up-regulation of RAC3 expression was also found in the

acute promyelocytic leukemic NB4 cells (data not shown). These results suggest not only that RAC3 can function as an RAR coactivator but also that its expression is autoregulated by the receptor-coactivator complex (Fig. 4C).

#### DISCUSSION

We show here that the nuclear receptor coactivator RAC3 utilizes a 140-amino acid domain to interact with liganded nuclear receptors and a C-terminal 162-amino acid domain to activate transcription, as well as to interact with CBP, and that the three C-terminal LXXLL motifs are sufficient for transcriptional activation. We demonstrate that RAC3, SRC-1, and TIF2 are expressed in a tissue-specific manner and that the expression of RAC3 can be up-regulated by retinoic acid. These findings have extended our and others' previous observations (42–44) and suggest that individual LXXLL motifs might contribute differently for receptor interaction and transcriptional activation. Our results suggest that RAC3 may enhance transcriptional activation of nuclear receptors through a combination of recruiting additional coactivators as well as autoregulation.

As RAC3 was initially characterized, several laboratories simultaneously reported the cloning of similar coactivators that are known as p/CIP (43), ACTR (44), and AIB1 (54). Comparison of these five sequences reveals that they are encoded by the same gene and that the differences should be due allelic variation. In particular, RAC3 and AIB1 share over 99.9% identity, with only two subtle differences, including a change in the length of the polyglutamine (poly-Q) tract (29 residues in AIB1 and 26 in RAC3). RAC3 and ACTR share



about 99.6% identity with several differences, including a 10-amino acid insertion at residues 321 and a 15-amino acid deletion between residues 902 and 918 of RAC3. RAC3 and the mouse p/CIP share only 77.6% identity with three major differences, including two unrelated gaps at amino acids 172–199 and 1296–1402, as well as the relative position of the poly-Q tract. Although the function of this poly-Q domain remains unclear, similar sequences are also found in many transcriptional regulators, and the elongation of the poly-Q tract appears to correlate with human disease (55), suggesting that the poly-Q tract within these three might have a functional implication yet to be established.

Our data suggest that the three N-terminal LXXLL motifs of RAC3 are likely involved in nuclear receptor interaction, consistent with recent studies with SRC-1 and p/CIP (43, 48), in which the individual motifs were found to be sufficient for nuclear receptor interaction. However, our attempts to demonstrate protein-protein interaction of each of the three RAC3 motifs with nuclear receptors were not successful (data not shown), suggesting that these motifs might act synergistically to stabilize the interaction if they indeed mediate such interaction. In contrast, we found that the three C-terminal motifs are sufficient to activate transcription. These results suggest that the conserved LXXLL consensus *per se* is insufficient to determine the function of these motifs and that specific structural constraints, perhaps defined by the neighboring residues, are important in determining their function.

It was recently shown that ACTR and SRC-1 both possess intrinsic HAT activity (31, 44). ACTR and SRC-1 preferentially acetylate histones H3 and H4, both in the free form and in the mononucleosome (31, 44). Comparison of the diacetyl H3 peptides suggests that Lys-9 and Lys-14 of histone H3 are the preferred sites for SRC-1 acetylation (31). Similarly, H3 is also a preferred substrate for ACTR (44), but the target residues remain unknown. Because histone acetylation has been correlated with gene activation (56, 57), these findings suggest that histone acetylation might be critical for gene activation by nuclear receptors. It remains unclear whether individual acetylases in the putative coactivator complex could modify histones in a redundant manner or whether different HAT enzymes are used for modification of distinct residues. In the latter case, such differential acetylation events might result in synergistic transcriptional activation. Alternatively, it was shown recently that the general coactivator CBP/p300 can also acetylate the sequence-specific tumor suppressor p53 and that such acetylation promotes DNA binding by p53 (32). It will be interesting to determine whether nuclear receptors can be acetylated and their DNA bindings regulated by these coactivators.

The expression patterns of RAC3, TIF2, and SRC-1 appear to be tissue- and cancer cell-specific, suggesting that the normal function of these coactivators might be limited to certain tissues and cells. The observed expression patterns of SRC family genes in the present study are different from earlier reports that used mouse tissues (37, 38, 41, 43). This might reflect a more selective expression of the SRC family in human tissues than in rodent tissues. The expression pattern of this family of genes in normal tissues is relatively similar, whereas the patterns between RAC3 and SRC-1 in cancer cells vary substantially. Lung carcinoma A549 cells appear to express none of the three coactivators, consistent with the lack of expression of these three genes in normal lung tissue. Correspondingly, lung carcinoma is resistant to the antitumor properties of retinoid treatment (58, 59). Interestingly, RAC3 and TIF2, but not SRC-1, are highly expressed in the Burkitt's lymphoma Raji cells, and all three SRC genes are highly expressed in the colorectal carcinoma SW480 cells. The mechanism of this over-

expression is unclear. It will be interesting to determine whether such overexpression contributes to neoplastic phenotype of these cancer cells and whether any of these coactivators are amplified in these cells, analogous to amplification of AIB1 in the breast cancer cells (54).

Autoregulation has long been established as a mechanism that contributes significantly to the high level of gene induction by retinoids (53). For instance, atRA treatment induces RAR $\beta$  gene expression, and an RAR response element was subsequently identified in the promoter region of this gene (60). Therefore, the binding of atRA to endogenous RAR is expected to activate expression of the receptor gene itself, thereby raising the level of the receptor and amplifying the effect of the ligand. This form of autoregulation is expected to play an important role in RA-dependent pattern formation and morphogenesis. It is now known that RAR is not the only factor responsible for such feedback control of gene activation. This study demonstrates that expression of the coactivator RAC3 is also increased by atRA treatment in at least two different RA responsive cancer cells. Thus, simultaneous enhancement of receptor and coactivator gene expression may both contribute to the formation an activator complex that stimulates gene expression upon ligand treatment. It remains to be determined whether the expression of RAC3 and other related genes can also be regulated by other hormones. In conclusion, this study suggests that transcriptional activation by nuclear receptor might be the result of a combined effect of coactivator recruitment and autoinduction of multiple effector genes.<sup>2</sup>

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<sup>2</sup> In light of the increasing number of members of this receptor coactivator family and the discrepancy in nomenclature throughout the literature, we would like to propose a unified nomenclature of members of this novel coactivator gene family. We propose to call this new family of coactivators the SRC gene family because SRC-1 was the first one cloned. Because RAC3 is the third member of this new family, we suggest the name SRC-3 for the RAC3/AIB1/ACTR/pCIP/TRAM-1 (61) gene and the name SRC-2 for the TIF2/GRIP1/NCOA-2 gene, while using SRC-1 for both SRC-1 and NCOA-1. An affixed "h" will be used for all human clones and an "m" for all mouse clones, such that the original SRC-1 will be called hSRC-1 and NCOA-1 will be called mSRC-1; TIF2 will be called hSRC-2 and GRIP1 and NCOA-2 will be called mSRC-2; and RAC3/AIB1/ACTR/TRAM-1 will be called hSRC-3 and p/CIP will be called mSRC-3.

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# The Receptor-associated Coactivator 3 Activates Transcription through CREB-binding Protein Recruitment and Autoregulation

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