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Nuclear localization of coactivator RAC3 is mediated by a bipartite NLS and importin α3 ☆

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Abstract

The nuclear receptor coactivator RAC3 (also known as SRC-3/ACTR/AIB1/p/CIP/TRAM-1) belongs to the p160 coactivator family, which are involved in several physiological processes and diseases. Here we have investigated how RAC3 is translocated into the nucleus and show that it is mediated through a bipartite NLS and importin α3. This bipartite NLS is located within the conserved bHLH domain, and its mutation abolished nuclear localization. The NLS is also sufficient to cause nuclear import of EGFP, and the activity requires basic amino acids within the NLS. RAC3 binds strongly to importin α3, which also depends on the basic amino acids. Functionally, RAC3 cytoplasmic mutant loses its ability to enhance transcription, suggesting that nuclear localization is essential for coactivator function. Together, these results reveal a previous unknown mechanism for nuclear translocation of p160 coactivators and a critical function of the conserved bHLH within the coactivator.

Keywords: Nuclear receptor coactivator; RAC3; Nuclear localization; Bipartite NLS; Importin α3; p160

Steroid/nuclear hormone receptors (NRs) are DNA-binding transcription factors that play important roles in many physiological processes and disease status such as cancer [1,2]. The activities of most NRs are regulated by cognate ligands, which ultimately control gene expression. In general, ligand binding induces a conformational change of the receptor, causing dissociation of corepressors and recruitment of coactivators. Among the known coactivators, p160 proteins are most extensively studied. These coactivators bind to ligand-activated NRs and enhance transcription by recruiting additional coactivators and basal transcription factors [3]. Three p160 coactivators have been identified so far, including SRC-1 [4], TIF2/GRIP1 [5–7], and RAC3/SRC-3/ACTR/AIB1/pCIP/TRAM-1 [8–12]. The p160 proteins contain several conserved functional domains that are involved in nuclear receptor interaction, transcriptional activation, or interactions with secondary coactivators [13–15]. Strikingly, the most conserved region is the N-terminal basic-helix–loop–helix-per-arnt-sim (bHLH-PAS) domain, which is also seen in other proteins such as BMAL2 [16] and ARNT [17]. Currently, little is known about the function of the bHLH-PAS domain although it has been shown to interact with several proteins such as the coiled-coil coactivator (CoCoA) [18] and the ankyrin repeats containing cofactor-1 (ANCO-1) [19].

Among p160 coactivators, RAC3 is important because it is amplified in breast and several other cancers [8]. In fact, RAC3 may play an important role in the development of breast cancer by forming a stable complex with estrogen receptor α [20]. Overexpression of RAC3 was shown in many malignancies, including esophageal squamous cell carcinoma [21], hepatocellular carcinoma [22], gastric cancers [23], pancreatic adenocarcinoma [24], and prostate

☆ *Abbreviations:* RAC3, nuclear receptor associated coactivator; NLS, nuclear localization signal; HEK 293, human embryonic kidney 293; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; AR, androgen receptor; GR, glucocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor.

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cancer [25,26]. Furthermore, transgenic mice overexpressing RAC3 develop spontaneous breast tumors [27], while RAC3 knockout suppresses v-Ha-ras-induced breast tumor initiation and progression [28]. These data strongly suggest that RAC3 is a proto-oncogene. Recent study further demonstrated that RAC3 regulates cell proliferation and transformation though binding of E2F1 [29,30]. It appears that RAC3 may control the G1 to S transition through controlling genes involved in initiation of DNA synthesis and replication. The activity of RAC3 and other p160 coactivators can be regulated by posttranslational modifications such as acetylation [31], phosphorylation [32–35], sumoylation [36], and ubiquitination [37–39]. For instance, p300/CBP can acetylate the lysine residues adjacent to a core LXXLL motif, disrupting the association of RAC3 with ERs [31]. RAC3 is localized primarily in the nucleus, although cytoplasmic localization has also been reported. It is assumed that RAC3 works in the nucleus to interact with various proteins to enhance transcription. However, the mechanism(s) that governs nuclear translocation of RAC3, or other p160 coactivators, has not been elucidated, nor the requirement of nuclear localization for the coactivator function of RAC3 been established.

In this study, we have investigated the regulatory signals that determine nuclear localization of RAC3, and the molecules that are responsible for piggybacking RAC3 into the nucleus. Indirect immunofluorescence microscopy analyses revealed that the conserved bHLH-PAS domain is directly involved in mediating nuclear translocation of RAC3. A bipartite nuclear localization signal (NLS) is identified within the bHLH domain of RAC3 and other p160 coactivators. Site-directed mutagenesis studies further demonstrated the involvement of two interdependent basic amino acid clusters. In addition, we show that nuclear import of RAC3 is mediated specifically by importin α3, which binds to the bipartite NLS. Finally, we provide strong evidence to support an essential role of nuclear localization for the transcriptional coactivation function of RAC3.

Materials and methods

Cell culture and reagents. COS-7 and HEK293 cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 5 μg/ml of gentamycin (Life Technologies, Grand Island, NY). Cell transfections were conducted with the standard calcium phosphate precipitate method. Appropriate numbers of cells (about 75% confluent) were plated in multi-well plates for about 12 h, and cells were then incubated with DNA–$CaPO_4$ precipitates for about 12 h. Transfected cells were washed three times with PBS, replaced with fresh media, and harvested about 36 h afterward. A cover glass was placed at the bottom of each well if the cells were for immunofluorescence studies. Primary antibodies used in this study were the anti-HA epitope mouse monoclonal (F-7, Santa Cruz Biotechnology) and rabbit polyclonal (Medical Biological Lab) antibodies. All cell culture and chemical reagents were of the highest quality available from various commercial sources.

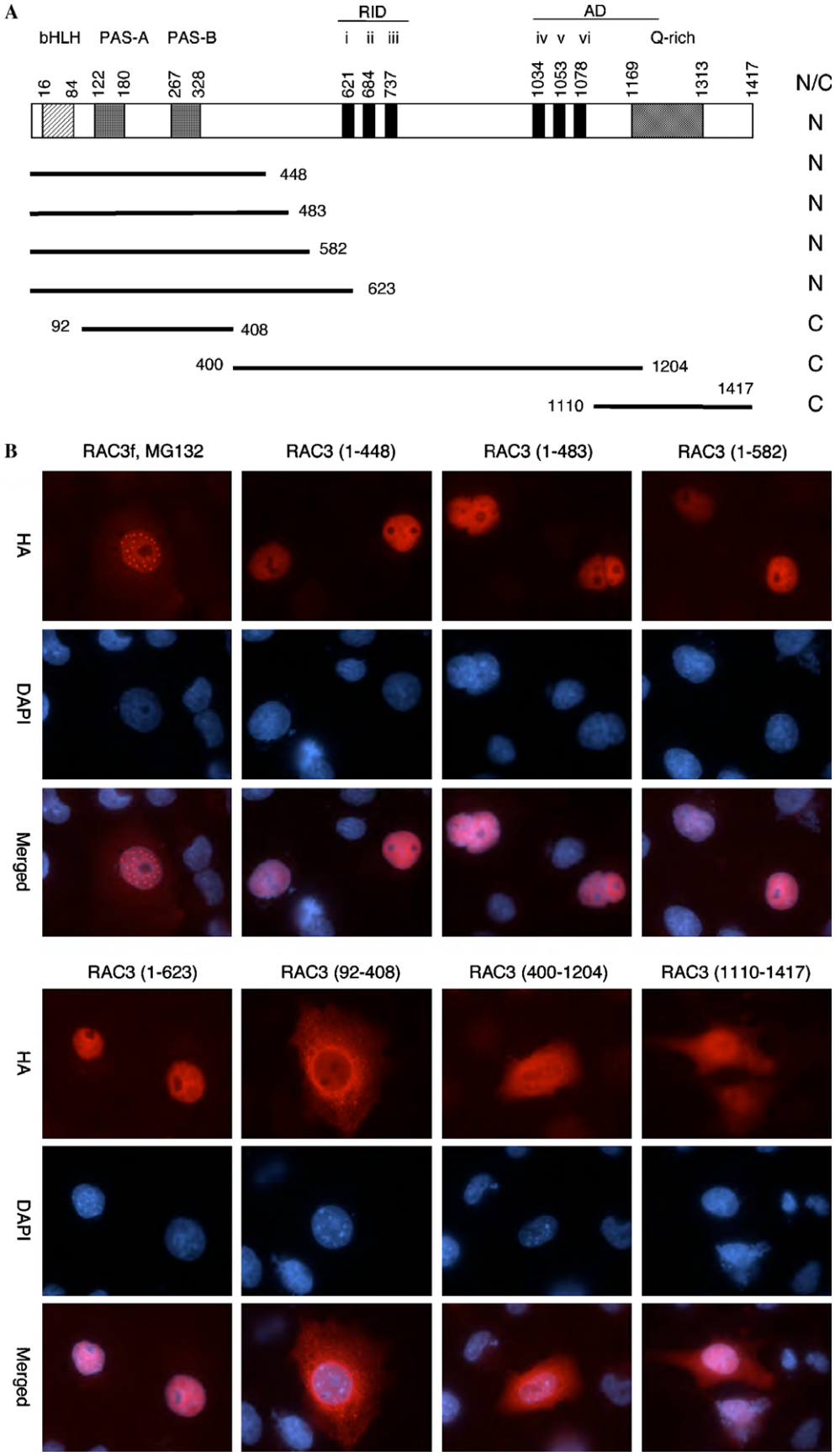
Plasmids. The human RAC3 cDNA used in this study was originally isolated in our laboratory from a human brain cDNA library [10]. The full-length RAC3, point mutants, and deletion mutants contain an N-terminal translation start site and a hemagglutinin (HA) tag (MDYPYDVPDY). Site-directed mutagenesis was conducted using QuickChange kit (Stratagene). Primer sequences are available upon request. Deletion mutants were generated by PCR amplification using Pfu with primer sets containing appropriate restriction sites for subcloning. EGFP fusion constructs were subcloned into the pEGFP-C1 vector (BD Bioscience). GST fusion constructs were subcloned into the pGEX-2T vector. All GST-importin constructs were kindly provided by Dr. Riku Fagerlund (National Public Health Institute, Helsinki, Finland) [40], except importin α4 (KPNA3), which was isolated from a human placenta cDNA library in our laboratory and subcloned into pGEX-2T vector. The yeast expression GAL4-DBD fusion constructs were subcloned into the pGBT9 vector. The pGAD-hQIP1 (importin α3) construct was kindly provided by Dr. Takemi Enomoto [41]. The yeast expressing GAL4 AD-importin α6 fusion construct was subcloned into the pACT2 vector.

Immunofluorescence microscopy. Cells were grown on cover glasses in 24-well plate and fixed in 4% paraformaldehyde or methanol/acetone (1:1) mixture on dry ice for 1 min. The fixed cells were processed for immunofluorescence staining as previously described [42]. After washing, cells were stained with rhodamine- or fluorescein-conjugated goat anti-mouse or anti-rabbit secondary antibodies. Cell nuclei were stained with DAPI. The processed cover glasses were mounted on slides with Pro-Long antifade reagents (Molecular Probe) and visualized with a Zeiss Axiovert 200 inverted epi-fluorescence microscope. The images were captured with Axiocam and analyzed by Axiovision software. Color images were exported from Axiovision and assembled using Canvas.

GST pull-down assay. GST pull-down assay was conducted according to a protocol described previously [43]. Briefly, 5 μg glutathione agarose–protein beads were incubated with 5 μl of in vitro translated ^{35}S-labeled protein with moderate shaking at 4 °C for 3 h in a binding buffer (20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40, 1 mM DTT, and 1 mg/ml BSA). The bound protein was washed three times with the binding buffer and beads were collected by low-speed centrifugation. The bound protein was eluted in SDS sample buffer before subjected to SDS–PAGE analysis and autoradiography.

Yeast two-hybrid assay. The yeast Y190 cells were cotransformed with indicated plasmids and 4 μg of single-stranded salmon sperm DNA according to the manufacturer's protocol (BD Biosciences, CA). Transformed yeast cells were spread on tryptophan and leucine selection plates and incubated at 30 °C for 2 days. Colonies were lifted off onto 3 mm Whatman paper and cell walls were broken at −80 °C for 10 min. Whatman papers were placed, colony side up, in a box containing Z-buffer and X-gal at 30 °C overnight and analyzed for β-galactosidase activity. For quantitative liquid assay, yeast colonies were picked from each plate

Fig. 1. Subcellular localization of RAC3 fragments. (A) Schematic representation of RAC3 fragments used in this study. The known domains and boundary amino acid residues are shown. bHLH, basic helix–loop–helix; PAS, Per-Arnt-Sim domain; RID, nuclear receptor-interacting domain; AD, transcriptional activation domain; i–vi, LXXLL motifs; Q-rich, glutamine-rich domain. (B) Indirect immunofluorescence showing subcellular localization of each RAC3 construct. COS-7 cells were transfected with indicated constructs and analyzed by immunofluorescence staining with anti-HA monoclonal antibody (red). The RAC3f (full-length) construct-transfected cells were treated with MG132. Without MG132 treatment, the RAC3f was barely visible. Cell nuclei were revealed by DAPI (4,6-diamidino-2-phenylindole) staining. The subcellular localization results are summarized at the right in (A). N, nuclear; C, cytoplasmic or pan-cellular staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)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RID
AD
bHLH
PAS-A
PAS-B
i ii iii
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Q-rich
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N/C
448
483
582
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92 408
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1110 1417
N
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N
N
C
C
C
B
RAC3f, MG132
RAC3 (1-448)
RAC3 (1-483)
RAC3 (1-582)
HA
DAPI
Merged
RAC3 (1-623)
RAC3 (92-408)
RAC3 (400-1204)
RAC3 (1110-1417)

and grown in tryptophan and leucine deficient media at 30 °C for 48 h. Yeast cells were then harvested in Z-buffer containing 0.27% β-mercaptoethanol and *O*-nitrophenyl-β-galactopyranoside as substrate and analyzed for β-galactosidase activity.

Transient transfection assays. HEK293 cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5 μg/ml gentamycin (Invitrogen). One day prior to transfection, cells were seeded in 12-well plates in phenol red free Dulbecco's modified Eagle's medium supplemented with 10% charcoal resin-stripped fetal bovine serum. Cells were incubated with DNA–$CaPO_4$ precipitates for about 12 h. Transfected cells were washed three times with PBS, and re-fed with fresh medium containing indicated treatments, and then harvested about 48 h afterward for luciferase and β-galactosidase assays as described previously [10,44].

Results

Localization of RAC3 fragments

To identify the primary amino acid sequence that determines subcellular localization of RAC3, we analyzed the distribution of a series of RAC3 fragments (Fig. 1A). These fragments contain a hemagglutinin (HA) epitope at their N-termini and were expressed in COS-7 cells followed by immunofluorescence microscopy analysis with anti-HA antibodies (Fig. 1B). Full-length

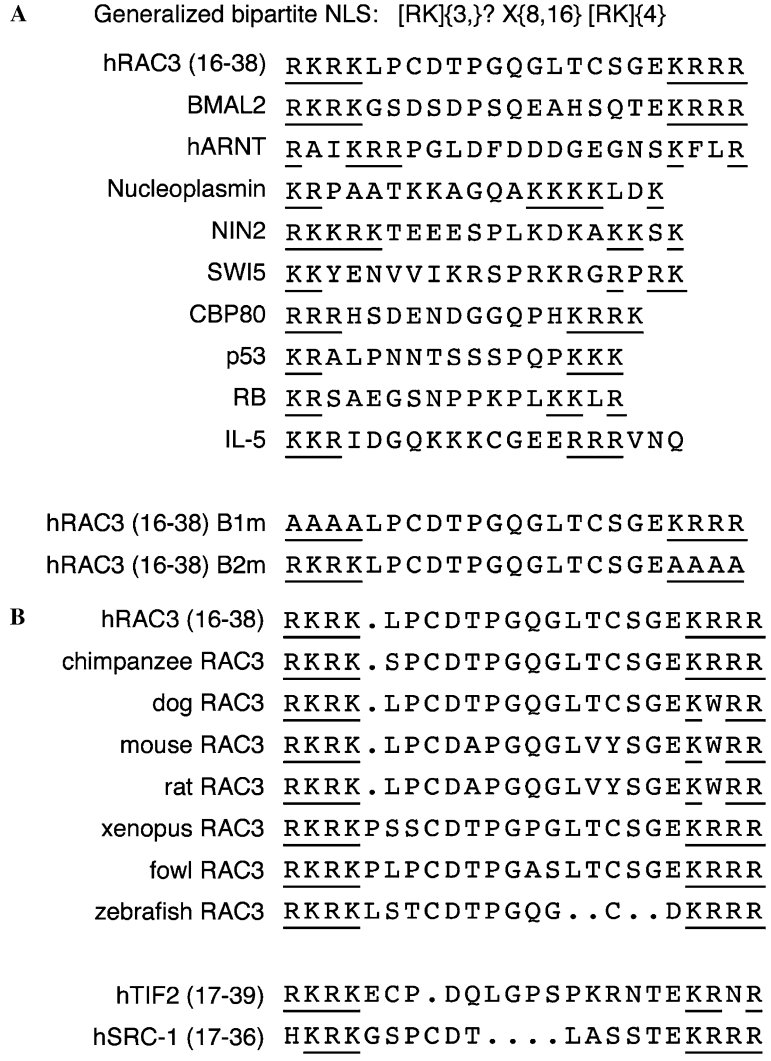

Fig. 2. Identification of a RAC3 bipartite nuclear localization signal. (A) Comparison of the predicted RAC3 bipartite NLS sequence with other bipartite NLSs. A generalized RAC3 consensus bipartite NLS sequence is shown at the top. BMAL2, a bHLH-PAS transcription factor; hARNT, human aryl hydrocarbon receptor nuclear translocator; N1N2, *Xenopus laevis* phosphoprotein; SWI5, *Saccharomyces cerevisiae* transcription factor; CBP80, CAP-binding protein 80; RB, retinoblastoma; IL-5, cytokine interleukin-5. The lower panel shows mutations of the two basic amino acid clusters, B1 and B2 within the RAC3 bipartite NLS. (B) Sequence alignment of the RAC3 bipartite NLS sequence across difference species, including human (GI: 2318006), chimpanzee (GI: 55652591), dog (GI: 73992554), mouse (GI: 6679026), rat (GI: 62646425), xenopus (GI: 23396770), fowl (GI: 50758717), zebrafish (GI: 68369802), and among members of the p160 family including hTIF2 and hSRC-1 (bottom panel). (C) Indirect immunofluorescence staining showing subcellular localization of B1 and B2 mutations (B1m and B2m) of the RAC3 bipartite NLS. COS-7 cells were transfected with HA-tagged RAC3 (1–448) or full-length RAC3 (RAC3f) with or without either B1 or B2 mutations (B1m or B2m). Transfected cells were analyzed by immunofluorescence staining with anti-HA polyclonal antibody (green). The RAC3f wild type (W.T.) construct-transfected cells were treated with MG132 to stabilize the protein. Cell nuclei were revealed by DAPI staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

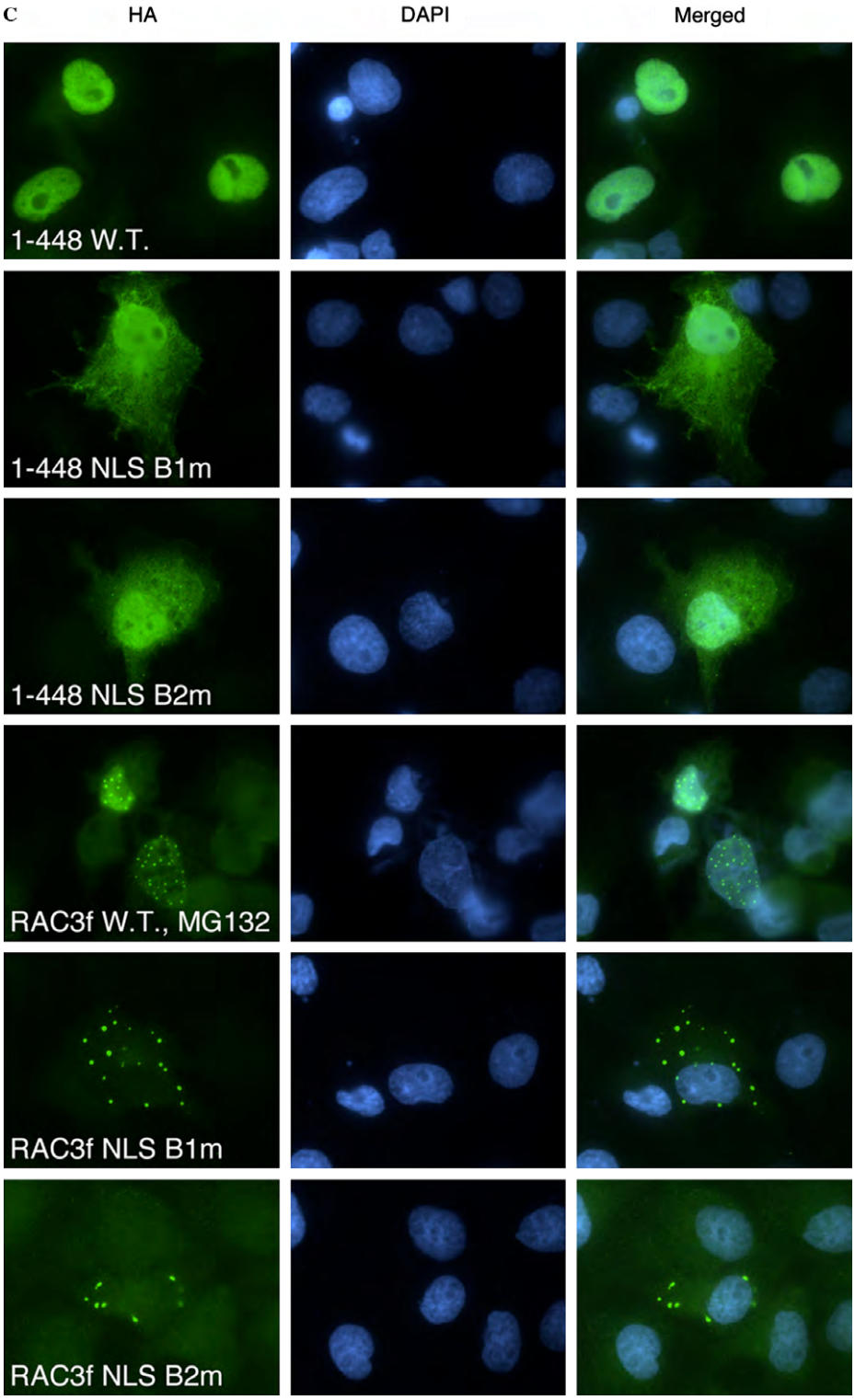

Fig. 2 (*continued*)

RAC3 (RAC3f) was found mainly in the nucleus in dot-like structures with a diffuse nucleoplasm staining. The expression of full-length RAC3 was apparent only after treatment with the proteasome inhibitor MG132 due to rapid turnover of the protein. Four N-terminal fragments of RAC3 containing amino acids 1–448, 1–483, 1–582, and 1–623 were all readily expressed and detected in the nucleus without MG132 treatment (Fig. 1B). In contrast, the RAC3 (92–408), the central (400–1204), and C-terminal (1110–1417) fragments were all localized in the cytoplasm or throughout the entire cells. These results suggest that nuclear localization signal of

RAC3 is located at the N-terminal, possibly within the first 91 amino acids.

Identification of a bipartite nuclear localization signal in RAC3

To determine potential nuclear localization signals within RAC3, we searched for NLS consensus sequence using various prediction programs. The PSORT II program predicted three simple NLS sequences at residues 16–19 (RKRK), 35–38 (KRRR), and 729–735 (PKKKENN), while the PredictNLS server identified a single bipartite NLS at amino acids 16–38 (RKRKLPCDTPGQGLTCS GEKRRR). The amino acids 16–38 sequence fits into a generalized bipartite NLS (Fig. 2A) and coincides with the basic region of the bHLH domain. It contains four positively charged amino acids at the N-terminus, followed by 15 intervening residues and a C-terminal basic domain. This sequence also matches well with other bipartite NLSs (Fig. 2A). Furthermore, this sequence is highly conserved in different species and among different members of p160 coactivators (Fig. 2B). Therefore, this sequence is the best NLS candidate for RAC3 and possibly other p160 coactivators. We excluded the sequence at residues 729–735 because the 400–1204 fragment did not accumulate in the nucleus (Fig. 1B).

To prove that amino acids 16–38 sequence is a bipartite NLS of RAC3, we mutated the two basic amino acid clusters, B1 and B2, by site-directed mutagenesis. The mutations (B1m and B2m) were created in the N-terminal1–448 fragment and the full-length protein. Both B1m and B2m mutations caused a significant accumulation of the 1–448 fragment in the cytosol compared to the wild type. Similarly, both mutations caused cytoplasmic accumulation of the full-length protein as well. Intriguingly, the full-length B1m and B2m mutants could be readily detected without MG132 treatment, suggesting that cytoplasmic localization of RAC3 renders the protein less sensitive to proteolytic degradation. These results strongly suggest that amino acids 16–38 sequence serves as a functional NLS for RAC3, and this activity requires both basic clusters.

RAC3 bipartite NLS is sufficient to mediate nuclear import

To further demonstrate that the RAC3 bipartite NLS is sufficient to mediate nuclear translocation, we generated several GFP-RAC3 fusions and analyzed their subcellular localizations. Whereas GFP is uniformly distributed in both

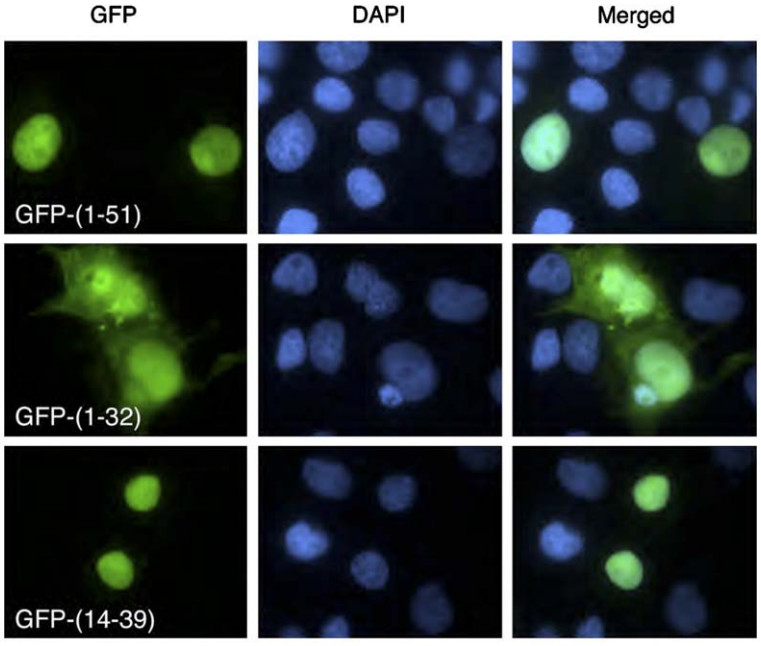

Fig. 3. The RAC3 bipartite NLS is sufficient for mediating nuclear import of GFP. The upper panel shows distribution of EGFP-RAC3 polypeptide fusions. COS-7 cells were transfected with indicated constructs, fixed in 4% paraformaldehyde, and visualized by epi-fluorescence microscopy. Cell nuclei were revealed by DAPI staining. The bottom panel shows the amino acid sequences of the RAC3 polypeptides fused to EGFP. The two basic amino acid clusters, B1 and B2 of the RAC3 bipartite NLS are underlined. The amino acid residues 14–39 are sufficient to mediate nuclear import of the EGFP.

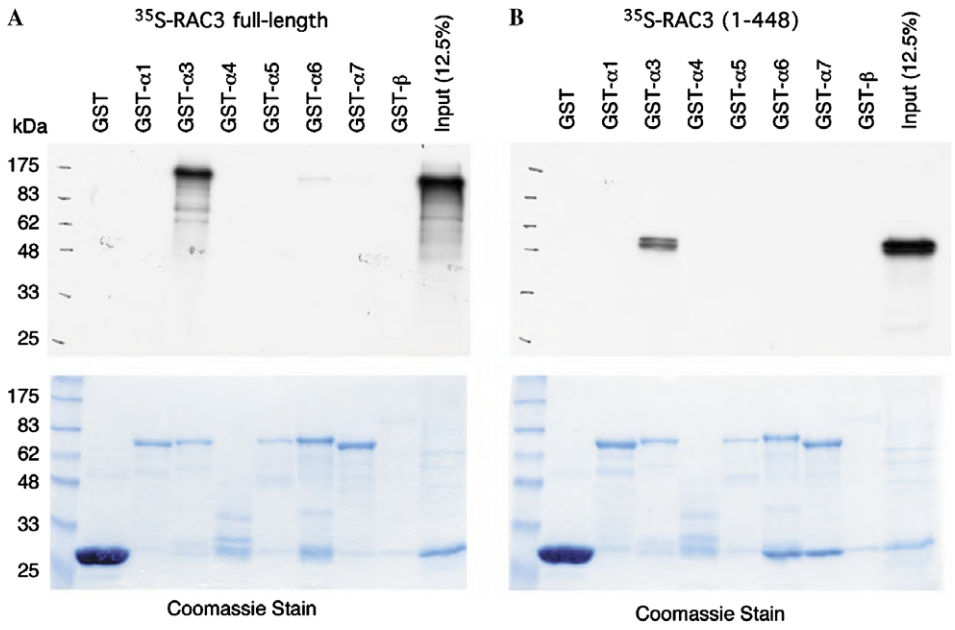

Fig. 4. RAC3 binds to importin α3. (A) Binding of full-length RAC3 specifically to GST-importin α3. GST and a series of GST-importin fusion proteins (GST-α1, 3, 4, 5, 6, 7, and β) were purified from BL21 bacteria cells. The amounts of GST proteins used in the pull-down assay are shown by Coomassie stain at the lower panel. The bound ^{35}S-RAC3 were detected by autoradiography (upper panel). RAC3 binds strongly to α3, and weakly to α6, but not to other importins. (B) Binding of RAC3 (1–448) fragment to GST-importin α3. Similar to full-length RAC3, the RAC3 (1–448) N-terminal fragment also bound strongly and selectively to importin α3.

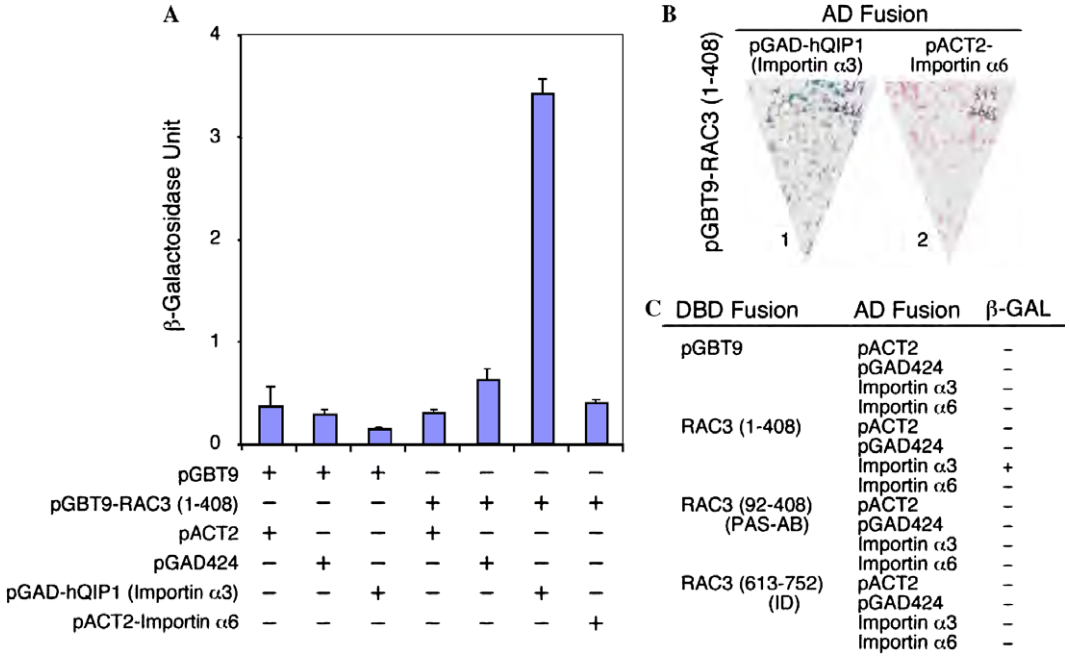

Fig. 5. RAC3 interacts selectively with importin α3 in yeast two-hybrid assay. (A) Liquid β-galactosidase assay showing specific interaction between Gal4 DBD-RAC3 (1–408) fusion with Gal4 AD-hQIP1 (importin α3) fusion. In this assay, the RAC3 (1–408) fragment showed no interaction with importin α6 or other control vectors. (B) Filter assay showing specific induction of β-galactosidase expression in the presence of pGBT-RAC3 (1–408) and pGAD-hQIP1. (C) Summary of the yeast two-hybrid filter assay data. RAC3 (1–408) interacts with importin α3 but not control vectors. Both RAC3 (aa 92–408) (PAS-AB) and (aa 613–752) (ID, nuclear receptor interacting domain) fragments showed no interactions with importin α3 or other vectors.

cytoplasm and nucleus, GFP-RAC3 (1–51) fusion was localized exclusively in the nucleus (Fig. 3). In contrast, GFP-RAC3 (1–32) fusion was localized in both cytoplasm and nucleus, indicating that the B2 region is required for NLS function. Furthermore, GFP-RAC3 (14–39) containing the minimal bipartite NLS sequence was localized exclusively in the nucleus. These results led us to conclude that this bipartite NLS of RAC3 is sufficient to mediate nuclear

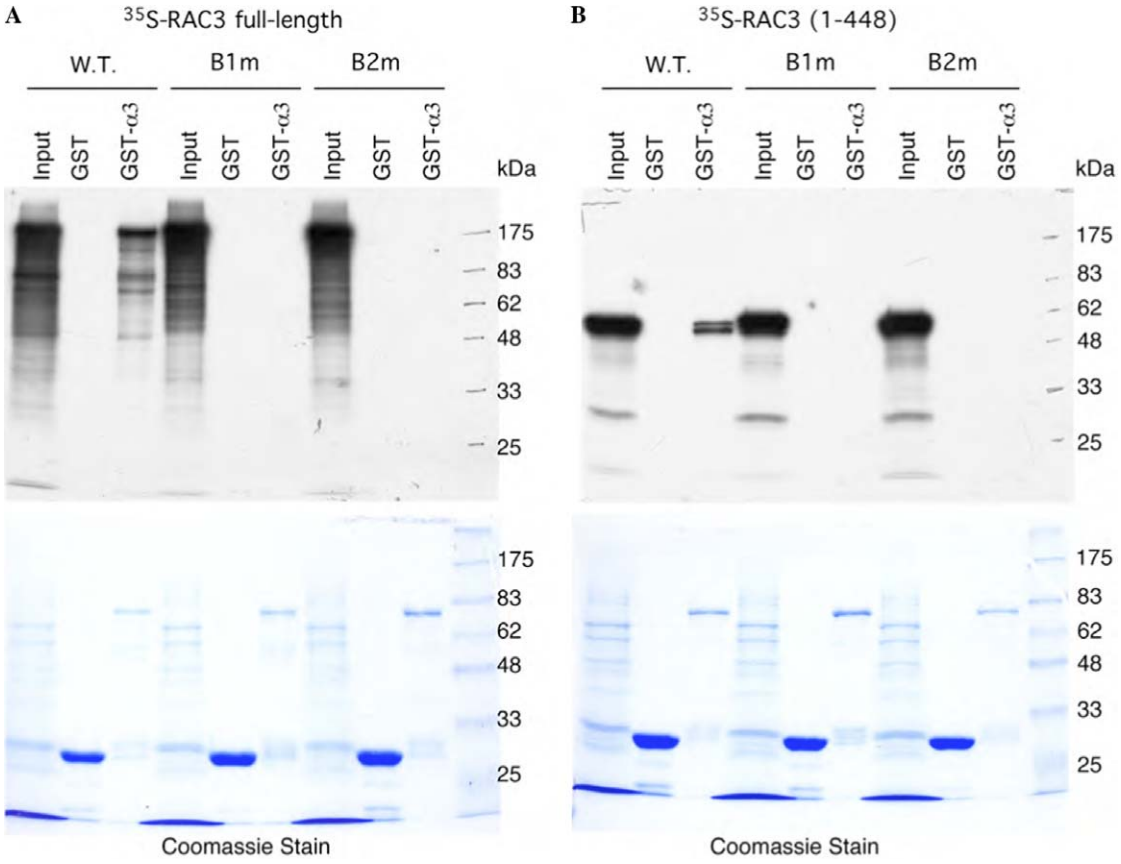

Fig. 6. Basic domain mutation blocks RAC3 binding to importin α3. (A) Basic domain mutations (B1m and B2m) block binding of importin α3 to full-length RAC3. The wild type (W.T.) and the B1 and B2 mutants of full-length RAC3 were synthesized in vitro in rabbit reticulocyte lysate and labeled with [^{35}S]methionine. The approximately equal amounts of each labeled proteins were incubated with GST or GST-importin α3 (α3) fusion. Bound ^{35}S-RAC3 proteins were detected by autoradiography shown in the upper panel. GST and GST-α3 proteins used in the assay were revealed by Coomassie stain in the lower panel. Both B1 and B2 mutations (B1m and B2m) block full-length RAC3 binding to GST-importin α3. (B) Similar as described in (A), except that ^{35}S-RAC3 (1–448) fragment was used as probe in the GST pull-down assay. Both B1 and B2 mutations block binding of RAC3 (1–448) fragment to GST-importin α3.

translocation of heterologous protein, further supporting its role in mediating nuclear import of the coactivator.

RAC3 binds to importin α3 through the bipartite NLS

The transportation of large proteins from cytoplasm into the nucleus requires an energy-dependent nuclear import mechanism (for reviews, see Refs. [45,46]). One pathway for nuclear import is mediated by the importin α/β heterodimer. To investigate how RAC3 is transported into the nucleus, we tested the binding of RAC3 to various importins. An in vitro GST-pull down assay was conducted to determine whether any of the known human importins bind to RAC3. Intriguingly, we found that both full-length and the N-terminal 1–448 fragment of RAC3 bound strongly only to importin α3 (Fig. 4), suggesting that importin α3 is the molecule responsible for nuclear import of the coactivator.

To further confirm the interaction between RAC3 and importin α3, we also conducted yeast two-hybrid assay. In this assay, RAC3 (1–408) fragment interacted strongly and specifically with importin α3, but not with importin α6 or other control vectors (Fig. 5). The RAC3-importin a3 interaction was measured by both quantitative liquid β-galactosidase assay (Fig. 5A) and the qualitative filter assay (Fig. 5B). In contrast, the RAC3 (92–408) and (613–752) fragments did not show detectable interaction. These data confirm that importin α3 binds specifically to the RAC3 N-terminal region both in vitro and in yeast cells.

Furthermore, we also determined whether the bipartite NLS is responsible for importin α3 binding. Strikingly, importin α3 showed no binding to either the B1m or the B2m mutants of full-length RAC3 (Fig. 6A), suggesting that importin α3 binds directly to the bipartite NLS. Similarly, both B1m and B2m mutations abolished binding of importin α3 to the RAC3 N-terminal 1–448 fragments. These results demonstrate that RAC3 binds selectively to importin α3 through the bipartite NLS.

Nuclear localization is essential for RAC3 coactivator function

RAC3 is known as a transcriptional coactivator for several nuclear receptors [10,30,44,47,48]. Here we wish to

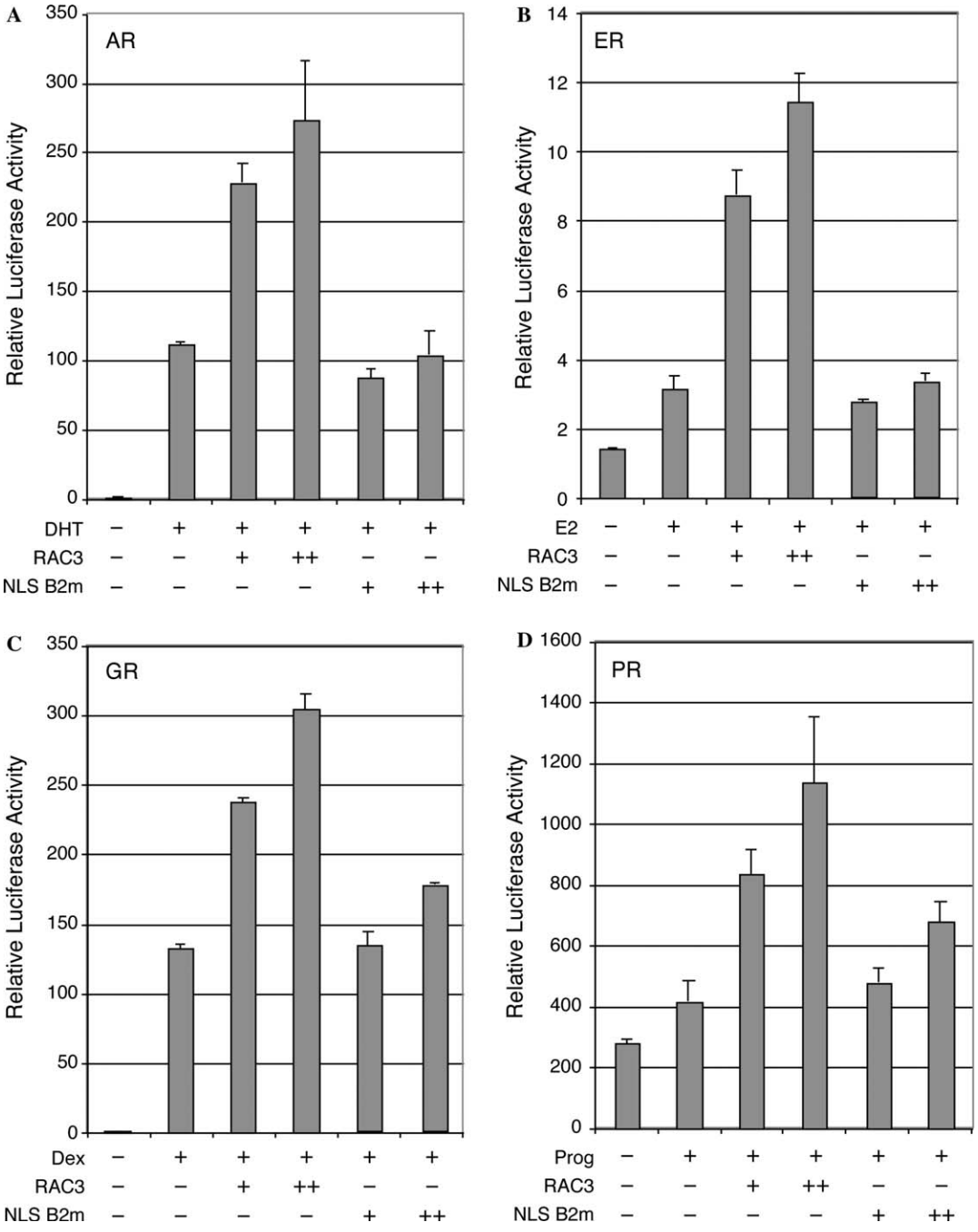

Fig. 7. Nuclear localization is essential for transcriptional coactivator function of RAC3. (A) RAC3 NLS mutation blocks its transcriptional coactivation on androgen receptor (AR). HEK293 cells were transiently transfected with either wild type full-length RAC3 or its bipartite NLS B2 mutant (B2m), together with human AR, mMTV-Luc reporter, and β-galactosidase control vector. Average relative luciferase activities were derived from three independent transfections. Where indicated, dihydroxytestosterone (DHT) was added at 10-nM concentration for approximately 48 h before the cells were harvested for luciferase and β-galactosidase assays. (B) RAC3 NLS mutation blocks its transcriptional coactivation on estrogen receptor (ER). The experiment was conducted similar to (A), except that human ERα and ERE-Luc reporter were used and the cells were treated with 10-nM 17β-estradiol. (C) RAC3 NLS mutation blocks its transcriptional coactivation on glucocorticoid receptor (GR). The experiment was conducted similar to (A), except that human GRα was used and the cells were treated with 1 nM dexamethasone. (D) RAC3 NLS mutation blocks its transcriptional coactivation on progesterone receptor (ER). The experiment was conducted similar to (A), except that human PR-B was used and the cells were treated with 1 pM progesterone.

establish that nuclear localization is indeed essential for the transcriptional coactivator function of RAC3. We compared the wild type and NLS mutant of RAC3 on several steroid receptor-mediated transcriptional activations (Fig. 7). Cotransfection of RAC3 led to a dose-dependent enhancement of the transcriptional activation by AR (Fig. 7A), ERα (Fig. 7B), GRα (Fig. 7C), and PR-B (Fig. 7D), consistent with prior studies [10,30,44,47,48].

In contrast, the NLS mutant failed to enhance transcriptional activation by these nuclear receptors. These results established nuclear localization as essential step for transcriptional coactivation by RAC3.

Discussion

p160 coactivators play important roles in regulating gene expression by functioning as transcriptional coactivators. We have investigated how RAC3, an important member of the p160 proteins, is translocated into the nucleus, and whether nuclear localization is essential for the coactivator function. We demonstrate that RAC3 contains a bipartite NLS sequence at amino acid residues 16–38. Two basic amino acid clusters within the NLS are both required for mediating nuclear localization, and this sequence alone is sufficient for targeting a heterologous protein into the nucleus. Interestingly, we discover importin α3 as the molecular carrier that interacts with RAC3 through the bipartite NLS. A precise correlation between importin α3 binding and nuclear localization of RAC3 is also established. Finally, we also demonstrate that nuclear localization is indeed essential for the transcriptional coactivation function of RAC3. This study characterizes the mechanism of nuclear translocation of the first member of the p160 coactivators, and establishes the importance of nuclear localization for the coactivator function.

Several N-terminal fragments of RAC3 were first found in the nucleus, while the central and C-terminal regions were in the cytoplasm (Fig. 1). These data first suggest that the N-terminal region of RAC3 determines nuclear localization. Since the first 91 amino acids are required for nuclear localization, and a consensus NLS is found at residues 16–38, we focused our investigation on establishing the function of this predicted NLS. By using several independent approaches, we firmly established the residues 16–38 motif as a functional bipartite NLS that decides nuclear localization of the coactivator. First, we found that the two basic amino acid clusters are both required for nuclear localization of RAC3 (Fig. 2), suggesting that this signal is a classic bipartite NLS. Second, we found that this bipartite NLS alone is sufficient to mediate nuclear import of a heterologous protein, and this ability requires both clusters of basic amino acids (Fig. 3). Third, this bipartite NLS apparently is directly involved in binding importin α3, a member of the importin family involved in nuclear import [49,50]. Finally, we showed that both basic amino acid domains are required for importin α3 binding, thus establishing a correlation between importin α3 binding and nuclear localization of RAC3.

There are six known human α importins. We identify α3 as the only importin isoform that binds significantly to RAC3. Therefore, importin α3 is likely the only carrier required for nuclear import of RAC3. It is conceivable that upon interaction with RAC3, importin α3 will then interact with importin β at the nuclear pore to form a cargo/importin α/β complex. This is followed by a GTP-dependent translocation of the importin α3–RAC3 complex through the nuclear pore into the nucleoplasm. Importin α isoforms display distinct substrate specificities, as most of them are expressed in the same cells and tissues. The binding affinity and specificity of importin α may be determined by the whole NLS binding groove as well as the flanking sequences of the NLS. It is currently unclear whether cells utilize only importin α3 or other available importins for nuclear translocation of RAC3. The revelation of a conserved bipartite NLS in the p160 coactivator suggests that this motif might also mediate nuclear translocation of other p160 proteins. Importin α3 contains 10 armadillo (arm) repeats, of which the N-terminal arm repeats 2–4 are considered as the major NLS binding site, whereas the C-terminal repeats 7–9 form the second NLS binding site. One importin molecule is able to use either the N- or C-terminal arm repeats for binding various NLS-containing proteins [51]. For example, importin α3 has recently been shown to use the N- and C-terminal NLS binding sites to bind p50 and p65 subunits of the NF-κB heterodimer, respectively [40]. Based on our results, we speculate that one importin α3 molecule may utilize its two NLS binding sites to cooperatively bind both basic amino acids clusters of the RAC3 bipartite NLS. Currently, we do not know which basic cluster binds to which NLS binding site of importin α3.

Interestingly, the RAC3 bipartite NLS identified here coincides with the basic amino acid region of the conserved bHLH-PAS domain. This suggests that one previously unknown function of the bHLH domain is to mediate nuclear translocation of RAC3. This NLS motif is highly conserved among RAC3 proteins from different species and among different p160 family proteins, suggesting that the same motif may be responsible for nuclear translocation of the other p160 proteins. Finally, we have established an essential role of nuclear localization for the transcriptional coactivation function of RAC3. We demonstrated that RAC3 cytoplasmic mutant lose their transcriptional coactivation function for AR, ER, GR and PR-mediated transcription (Fig. 7). Since the cytoplasmic mutant contains intact nuclear receptor interacting domain and transcriptional activation domain, the loss of transcriptional coactivation function should be due to lack of nuclear localization. The N-terminal bHLH-PAS domain of p160 proteins has been reported to be essential for interaction with several other proteins including CoCoA [18] and mMS19 [52], as well as for transcriptional coactivation of nuclear receptors [18]. Therefore, the bHLH-PAS domain of p160 protein may have multiple functions. This study unexpectedly reveals a new and essential function of the bHLH domain in mediating interacting with importin α3 and nuclear translocation of the coactivator.

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