Nuclear localization signal in a cancer-related transcriptional regulator protein NAC1

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Abbreviations: BEN, BANP, E5R and NAC1; BTB, broad Complex, Tramtrack and Bric à brac; GADD45GIP1, growth arrest and DNA damage-inducible protein 45 gamma-interacting protein 1 (GFP), green fluorescent protein; mC, mCherry; NAC1, nucleus accumbens-associated protein 1; NLS, nuclear localization signal; sRNA, small interfering RNA; ZF, zinc finger.

Introduction

Nucleus accumbens-associated protein 1 (NAC1) has potential oncogenic properties and participate in regulatory networks for pluripotency. Although NAC1 is described as a transcriptional regulator, the nuclear import machinery of NAC1 remains unclear. We found, using a point mutant, that dimer formation was not committed to the nuclear localization of NAC1 and, using deletion mutants, that the amino-terminal half of NAC1 harbored a potential nuclear localization signal (NLS). Wild type, but not mutants of this region, alone was sufficient to drive the importation of green fluorescent protein (GFP) into the nucleus. Bimax, a synthetic peptide that blocks the importin α/β pathway, impaired nuclear localization of NAC1 in cells. We also used the binding properties of importin to demonstrate that this region is an NLS. Furthermore, the transcriptional regulator function of NAC1 was dependent on its nuclear localization activity in cells. Taken together, these results show that the region with a bipartite motif constitutes a functional nuclear import sequence in NAC1 that is independent of NAC1 dimer formation. The identification of an NAC1 NLS thus clarifies the mechanism through which NAC1 translocates to the nucleus to regulate the transcription of genes involved in oncogenicity and pluripotency.

Complex, Tramtrack and Bric à brac (BTB)/Poxvirus and Zinc finger domain and a carboxyl-terminal BANP, E5R and NAC1 (BEN) domain. NAC1 homodimerizes through its BTB domain, in which the interaction interfaces between subunits resembling those in transcription factors that contain BTB (12). NAC1 lacks a characteristic zinc finger (ZF) or basic leucine-zipper DNA-binding domain and thus differs from most other BTB-domain transcription factors. The BEN domain was identified by computational analysis and it might mediate protein–DNA and protein–protein interactions during chromatin organization and transcription (13).

NAC1 is a direct binding partner of Nanog, a core factor for the mammalian pluripotency program (14,15). NAC1 transcriptionally regulates gene expression in pluripotency factors including Nanog, Oct4 and Sox2, and participates in transcriptional regulatory networks that are critical to establish and/or maintain embryonic stem cell pluripotency (14,16,17).

Materials and methods

Materials

The following commercial antibodies were used: monoclonal anti-FLAG (M2) and anti-β-actin (AC-15) (Sigma, St Louis, MO, USA); anti-mCherry (mC) (Clontech, Mountain View, CA, USA); anti-green fluorescent protein (GFP) (BioAcademia, Osaka, Japan); anti-GADD45GIP1 (Novus Biologicals, Littleton, CO, USA); polyclonal anti-importin α3 and anti-importin α4 (Bethyl Laboratories, Montgomery, TX, USA); anti-GFP (BioAcademia, Osaka, Japan); horseradish peroxidase-HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA); and Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Carlsbad, CA, USA).

Plasmid construction

Frames of the human NAC1 gene encoding residues 2–527 (full-length, removal of the first methionine), 2–250, 2–129 and 2–125 were cloned into altered version of pMXs, pMXs-FHG that contained Kozak sequence, FLAG-tag, HA-tag and enhanced GFP (EGFP) at 5’ in the multi-cloning sites (BamHI-EcoRI/Xhol-NotI-Sall) by PCR using pcDNA6-V5/NAC1 as template (1). The gene identification numbers are listed in Supplementary Table 1 available at Carcinogenesis Online. NAC1 Y86A mutant was created by standard double PCR mutagenesis using the primers NAC1 Y86A-S and Y86A-AS. The primer sequences are listed in Supplementary Table 2 available at Carcinogenesis Online. Human NAC-2 gene was cloned into pMXs-FHG by PCR using IMAGE clone 4654320 as template.

To evaluate the nuclear localization signal (NLS) activity of peptide sequences in mammalian cells, double-stranded oligonucleotides encoding peptides including NAC1 (180–207), NAC1 (170–217), Xenopus nucleoplasm (155–172) and Bimax1 (RRRRPRKRPLEWEDEEPPRRKRWL) were inserted into pMXs-FHG.

The cDNA sequences of the full-length human importin α1, α3, α4 and α6 were PCR amplified using oligo(dt)-primed first-strand cDNAs from human fetal brain (Nippon Gene; Toyama, Japan) (18). For GST fusions, the importin α1 (70–529), α3 (65–521), α4 (65–521) and α6 (70–539) cDNAs were cloned into pGEX-6P-1 (GE Healthcare, Piscatawy, NJ, USA) (19). Importin α3 mutants at the major NLS binding site (W179A/N183A) were created by standard double PCR mutagenesis using the primers W179A/N183A-S and W179A/N183A-AS.

For mC fusions, the mC coding region derived from pMXs-mc (20) was cloned into a modified pET-28a (Novagen, Madison, WI, USA) with a hexa-histidine-tag and TEV cleavage site at the amino-terminus and with multi-cloning sites (BamHI-EcoRI/XhoI-HindIII-NotI) at the C-terminus, to generate pET28-His-TEV-mC. Double-stranded oligonucleotides encoding NLS peptides including NAC1 (180–207) and Xenopus nucleoplasm (155–172) were inserted into pET28-His-TEV-mC. The NAC1 (180–207) mutants K183A/R184A (mut-N), R201A/K202A/K205A (mut-C) and K183A/R184A/R201A/K202A/K205A (mut-N + C) were created by the corresponding double-stranded oligonucleotides.

All PCR-amplified cDNA products were fully sequenced with 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) to confirm mutations and to verify the absence of secondary point mutations.

Abbreviations: BEN, BANP, E5R and NAC1; BTB, broad Complex, Tramtrack and Bric à brac; GADD45GIP1, growth arrest and DNA damage-inducible protein 45 gamma-interacting protein 1 (GFP), green fluorescent protein; mC, mCherry; NAC1, nucleus accumbens-associated protein 1; NLS, nuclear localization signal; sRNA, small interfering RNA; ZF, zinc finger.

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Monoclonal antibody generation

For making the anti-NAC1 mouse monoclonal antibody 9.27, we immunized mice with recombinant GST-tagged full-length NAC1, and serum titers were monitored. Cells were transfected with plasmids expressing GST-fused NAC1 and then harvested for Western blotting. To detect the recombinant NAC1, 10% fetal bovine serum (Cell Culture Technologies, Gravesano, Switzerland) was used as the blocking solution. The mouse monoclonal antibody 9.27 was used to detect NAC1, and the secondary antibody was horseradish peroxidase-conjugated goat anti-mouse antibody (Invitrogen). Signals were visualized using eCL IP Kit (Atto-Science, Tokyo, Japan).

Cell culture, transfection, and retroviral infection

All adherent cells and hybridomas were grown in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) and RPMI1640 medium, respectively, supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gravesano, Switzerland). All adherent cells were transiently transfected with each plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Retrovirus production has been described (20). Overexpression of NAC1 proteins gives rise to the formation of dense body-like structures in the nucleus (1,12). Only GFP-positive cells expressing the lower detectable amounts of fusion protein were isolated by the FACSARis III using FACS Daiva software (BD, Franklin Lakes, NJ, USA).

RNA interference and complementation

Steadh small interfering RNA (siRNA) against NAC1 (#1, 5′-CCGGCUCAUAUGUAACCGUGAAUU-3′; #2, 5′-CAACGUUAGGAGACGUGAU-3′) were purchased from Invitrogen. siRNA was transfected into cells using Lipofectamine RNAMax (Invitrogen) according to the manufacturer’s instructions.

The pMXs-IG (22) construct that renders the corresponding NAC1 mRNA insensitive to the siRNA#1 with no effect on the amino acid sequence of expressed NAC1 protein was created by standard double PCR mutagenesis using the primers NAC1-siR-3S and NAC1-siR-4AS.

Immunofluorescence microscopy

Cells were simultaneously fixed and permeabilized for 10 min with 3.7% formaldehyde and 0.2% Triton X-100 in PBS, and then washed 3 times with PBS and then blocked for 30 min with 5% skimmed milk in PBS. After the blocking, cells were incubated with primary antibodies for 1 h at room temperature. After four washes with PBS, secondary antibodies incubations were performed for 1 h at room temperature and the coverslips were mounted (mounting medium; Vector Laboratories, Burlingame, CA, USA) with DAPI. Cells were observed under a confocal microscope FV1000 (Olympus, Tokyo, Japan).

To evaluate the NLS activity of Emerald GFP-fused peptides, transfected cells were fixed with 3.7% formaldehyde with/without membrane permeabilization and then the Emerald GFP signals were observed under a confocal microscope FV1000.

Time-lapse recording using confocal microscopy

Confocal time-lapse fluorescence images were acquired using an FV1000 laser scanning confocal unit coupled to an inverted microscope (model IX-71, Olympus) equipped with a 1.4 NA UPLSAPO 100X oil objective and Fluoview software (Olympus). Cells were maintained at 37°C and analyzed on a 35 mm glass-based dishes (IWAKI, Tokyo, Japan) in CO₂-independent medium (Invitrogen) to avoid medium acidification in the CO₂-free atmosphere. Using GFP or mTomato filter sets and DIOC optics, 15 optical sections were collected at 1 μm step size every 90 s. Each series was projected onto a single z-plane. Time-lapse figures and movies were created using Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

Tissue samples and immunohistochemistry

Ovarian cancer patients underwent surgery at the Department of Obstetrics and Gynecology at Shumane University Hospital. Paraffin-embedded tumor tissues were obtained from the Department of Pathology. Diagnosis was based on conventional morphologic examination of hematoxylin-stained sections. Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shumane University). Immunohistochemistry was done by deparaffinized sections with autolavation for 10 min. After antigen retrieval in a sodium citrate buffer (pH 6.0), slides were incubated overnight at 4°C with the NAC1 antibodies at a dilution of 1:100 and an EnVision peroxidase kit (DAKO, Glostrup, DK, Denmark). The slides for all the samples were evaluated with a light microscope by two pathologists.

Expression and purification of recombinant proteins

Importins fused with GST were expressed in Escherichia coli BL21 by induction with 0.1 mM IPTG followed by sequential incubation each for 2 h at 37°C. mC proteins fused with various NLS sequences were expressed in BL21(DE3) by induction with 0.1 mM IPTG followed by sequential incubation each for 2 h at 37°C. Purification of proteins was carried out as described previously (23).

Importin binding assay

mC fusion proteins of 2 μg were allowed to bind in extraction buffer to Sepharose-immobilized GST-importin α at 4°C for 2 h, followed by washing 3 times with the buffer. Sepharose beads were dissolved in 10 μl of 2× Laemmlı sample buffer, and the proteins were separated on 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue.

Bioinformatics

NAC1 sequences containing NLS were identified by manually analyzing all entries in the GenBank (http://www.ncbi.nlm.nih.gov). Sequences were aligned with CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-e.html).

Results

Subcellular localization of NAC1

We developed anti-NAC1 monoclonal antibody 9.27 that selectively recognize FLAG-HA-EGFP (FHG)-tagged NAC1 fusion protein expressed in HeLa cells, but not the sole family protein FHG-NAC2 that contains both BTB and BEN domains (Figure 1A and Supplementary Figure 1 available at Carcinogenesis Online). The antibody detected endogenous NAC1 expression in HeLa cells (Figure 1B). As further evidence that the signal reactive with the antibody was authentic NAC1, the antibody failed to detect any bands in NAC1-negative TOV-21G (a human ovarian cancer cell line) and detected a very faint band in NAC1 knockdown HeLa cells (Figure 1B, middle and right panels). Immunofluorescence or immunochemical analysis using the antibody revealed that endogenous NAC1 almost exclusively localized with a fine granular pattern in the nucleus excluding nucleoli in HeLa cells (Figure 1C) and cancerous human tissues (Figure 1D) as described (6,9). Next, we used live-cell imaging to monitor the subcellular distribution of full-length NAC1 fused at the C-terminal to EGFP in HeLa cells (Figure 1E and Supplementary video 1). During interphase, GFP-NAC1 localized throughout the nucleoplasm. At nuclear envelope breakdown, GFP-NAC1 dispersed throughout the mitotic cytoplasm without specific localization (e.g. in chromosomes or centrosomes). As the nuclear envelope reform after mitosis, GFP-NAC1 gradually accumulated in the nucleus. These immunofluorescence and time-lapse microscopy studies demonstrate that NAC1 is a nuclear protein.

Nuclear localization signal of NAC1

Nuclear localization lies at the heart of the transcriptional regulator, but the regulation of nuclear NAC1 import is poorly understood. To uncover determinants that are responsible for nuclear NAC1 localization, we generated a series of NAC1 deletion mutants fused with GFP and investigated their subcellular distribution using fluorescence confocal microscopy. HeLa cells express endogenous NAC1 (Figure 1A–C) and the BTB domain mediates homodimerization (12). To eliminate the effect of endogenous NAC1 on the subcellular distribution of GFP-tagged exogenous NAC1, we created the point mutation Y86A, in which a conserved tyrosine residue within the BTB domain of NAC1 was replaced with alanine to confer resistance to importin binding (20). As further evidence that the signal reactive with the antibody was authentic NAC1, the antibody failed to detect any bands in NAC1-negative TOV-21G (a human ovarian cancer cell line) and detected a very faint band in NAC1 knockdown HeLa cells (Figure 1B, middle and right panels). Immunofluorescence or immunochemical analysis using the antibody revealed that endogenous NAC1 almost exclusively localized with a fine granular pattern in the nucleus excluding nucleoli in HeLa cells (Figure 1C) and cancerous human tissues (Figure 1D) as described (6,9). Next, we used live-cell imaging to monitor the subcellular distribution of full-length NAC1 fused at the C-terminal to EGFP in HeLa cells (Figure 1E and Supplementary video 1). During interphase, GFP-NAC1 localized throughout the nucleoplasm. At nuclear envelope breakdown, GFP-NAC1 dispersed throughout the mitotic cytoplasm without specific localization (e.g. in chromosomes or centrosomes). As the nuclear envelope reforms after mitosis, GFP-NAC1 gradually accumulated in the nucleus. These immunofluorescence and time-lapse microscopy studies demonstrate that NAC1 is a nuclear protein.

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We could not identify NLS motifs within NAC1 by a series of computer searches using such tools as PROSITE (http://prosite.
The classical NLS consists of either one (monopartite) or two (bipartite) stretches of basic amino acids. Visual examination of a cross-species comparison of amino acid sequence alignment of NAC1 revealed two clusters of basic amino acids (KR at one end and RKXAK/R at the other) separated by ~13–21 amino acid linker within the region (Figure 3A). To determine whether this region was sufficient to mediate nuclear import, the NAC1 polypeptide (180–207 or 170–217), both comprising the putative NLS of NAC1 (183–205), was appended to GFP (see schematics in Figure 3B, above panel) and we assessed the localization of the peptide containing fusion proteins compared with GFP alone. Proteins of the appropriate size were expressed in transfected HeLa cells (Supplementary Figure 2 is available at Carcinogenesis Online). Although the molecular sizes...
of GFP and GFP-NAC1 (180–207) are less than the diffusion limit (~50 kDa) of the nuclear pore complex (25), our prediction was that the NLS motif, if functionally competent, would enhance localization of the fusion protein to the nucleus. GFP-NAC1 (180–207) expressed cells enhanced localization of the fusion proteins to the nucleus (Figure 3B). These results were consistent with those obtained with

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**Fig. 2.** Subcellular localization of deleted forms of NAC1. (A) A schematic representation of deleted forms of NAC1. (B) Wild-type, not mutated form (Y86A) of NAC1 homodimerized with NAC1. HeLa cells were transfected with wild-type or mutated form (Y86A) of GFP-tagged full-length NAC1, together with mC-tagged NAC1. After 48 h, cells were harvested, and immunoprecipitations were performed with anti-GFP antibody. The precipitates were analyzed by immunoblot probed with anti-mC antibody. (C) HeLa cells were transfected with GFP alone or with GFP fused to either full-length or deletion mutants of NAC1 and fixed after 48 h. Images were obtained under a 473 diode laser. All results are representative of three independent experiments. Bars, 10 μm.
Nuclear translocation of NAC1 involves importins

Because the molecular size of NAC1 (60kDa) is much larger than the diffusion limit (~50kDa) of the nuclear pore complex (25), we expected its translocation into the nucleus to be mediated by a carrier-dependent import mechanism. Cargo proteins that contain NLS(s) are recognized, in the cytoplasm, through their NLS(s) by the adaptor proteins termed importins α and/or β, which upon binding the cargo proteins result in a complex that is then ferried through the nuclear pore complex in the nuclear membrane into the nucleoplasm (26,27). Bimax1 is a recently developed synthetic peptide that blocks the importin α/β pathway by an extremely high affinity for importin αs (18).

To assess the impact of Bimax1 on nuclear localization of NAC1, mC-NAC1 expressing HeLa cells were transfected with GFP or GFP-Bimax1. Live cell microscopic analysis revealed that GFP-Bimax1 impaired nuclear localization of mC-tagged NAC1 in cells that exited from mitosis; it had no effect in cells before nuclear envelope breakdown (Figure 4, left panels and data not shown), probably due to the absence of capability of Bimax1 to extrude nuclear proteins to cytoplasm and/or the lack of a nuclear export signal in NAC1. Time-lapse imaging clearly confirms the above-mentioned findings (Supplementary Figure 3 and Video 2A and B). As would be predicted, GFP expression alone had no effect even when cells exited from mitosis (Figure 4, right panels). Because Bimax1 is a strong and specific binder for the importin α family (18), these results suggest that Bimax1 competed effectively against importin αs for binding to NAC1 that had an intact NLS motif.

Importin binding as evidence for the presence of NLS in NAC1

The human genome encodes seven importin αs that fall into three phylogenetically distinct groups (Supplementary Figure 4A and B). Human importin α1, α3 and α6 are representatives of human importin subgroups. In the next approach, we used importin binding properties to demonstrate the presence of NLS in NAC1. We used GST pull-down assays to confirm binding between mC-tagged NAC1 (180–207) and GST-tagged importin α1, α3 and α6 proteins and detected importin-bound polypeptides by staining with Coomassie blue (Figure 5A).

The NAC1 (180–207) polypeptide was preferentially detected in the pulled down complexes associated with importin α3 (Figure 5A, lane 4), but not with importin α1 or α6. Note that mC-tagged NAC1 (180–207) was not a degradation product of GST-tagged importin α3 (see Figure 5B, lane 4 and C). Under identical conditions, GST protein per se did not bind with NAC1 (180–207) polypeptide (Figure 5A, lane 2), indicating that importin mediated the direct binding of GST-importin α3 with NAC1.

**Fig. 3.** Nuclear localization signal of NAC1. (A) Multiple sequence alignment of putative bipartite NLS of NAC1s. Source and corresponding gene identification (gi) numbers are indicated. Human, Homo sapiens (gi: 16418383); chimpanzee, Pan troglodytes (gi: 114675683); orangutan, Pongo abelii (gi: 297703774); monkey, Macaca mulatta (gi: 109126540); opossum, Monodelphis domestica (gi: 126323172); horse, Equus caballus (gi: 149756840); cattle, Bos taurus (gi: 300797615); mouse, Mus musculus (gi: 268872821); rat, Rattus norvegicus (gi: 19705547); platypus, Ornithorhynchus anatinus (gi: 149635584); dog, Canis lupus familiaris (gi: 73986836); puffer fish, Tetraodon nigroviridis (gi: 47214905); zebrafish, Danio rerio (gi: 189517185). Alignments were developed using CLUSTALW. Color coding reflects conservation of amino acid types—for example, yellow, NAC1 polypeptide (170–217) (data not shown). In contrast, replacing one or both clusters of basic amino acids of the putative NLS motif with alanine residues eliminated nuclear accumulation (Figure 3B). Furthermore, mutating the putative NLS motif in wild-type or Y66A-based full-length NAC1 results in failure to localize NAC1 to the nucleus (Figure 3C). Thus, these results indicate that NAC1 (180–207) polypeptide functions as potential NLS in cells.
Asn183) NLS binding site is replaced with Ala. A mutated polypeptide terminal (Arg 201, Lys 202 and Lys 205) stretches of basic amino acids mutated NAC1 (180–207) in which the initial (Lys 183 and Arg 184) or important for binding importin, we performed binding assays with α NLS binding site of importin α3 was not detected in the major NLS binding site of importin α3 (180–207) peptide and importin α3 (Figure 5B, lane 9), indicating that the NAC1 (180–207) polypeptide binding was specific for the region that contains the NLS and mediated through the major NLS binding site of importin α3.

To further confirm that the NLS sequence in NAC1 (180–207) is important for binding importin, we performed binding assays with mutated NAC1 (180–207) in which the initial (Lys183 and Arg184) or terminal (Arg200, Lys202 and Lys205) stretches of basic amino acids were replaced with Ala separately or together (mut-N, mut-C and mut-N + C, respectively). None of the mutated polypeptides were detected in association with NAC1 (180–207) polypeptide (Figure 5B, lane 9), indicating that the NAC1 (180–207) polypeptide binding was specific for the region that contains the NLS and mediated through the major NLS binding site of importin α3.

Fig. 4. Expression of Bimax1 impairs nuclear localization of NAC1. mC-tagged NAC1 expressing HeLa cells were infected with retrovirus carrying with GFP (right panel) or GFP-tagged Bimax1 (left panel). Live cell microscopy was then performed. Bars, 10 µm. See Supplementary Figure 3 and Video 2, A and B.

Discussion

We performed GST pull-down assays to examine binding between GST-tagged importin α3 and mC, mC-tagged NAC1 (180–207) and nucleoplasmin (NLS, 155–172) polypeptides. NAC1 (180–207) tagged with mC was detected to a level equivalent to that of a positive control nucleoplasmin (NLS) polypeptide in the pulled down complexes associated with importin α3, confirming binding between NAC1 (180–207) polypeptide and importin α3 (Figure 5B, lanes 5 and 6).

Importin α3 contains two NLS binding pockets (Figure 5E). To determine whether the binding of NAC1 polypeptide with importin is mediated through these NLS binding pockets, we performed binding assays with a mutated importin α3 in which the major (Trp179 and Arg184) NLS binding site of importin α3 has the closest paralogs of importin α4; as α3 could pull-down NAC1 (180–207) peptide (Figure 5D).

Thus, we demonstrated that NAC1 has an authentic bipartite nuclear localization sequence through its ability to interact with the importin α3/4 proteins of the nuclear transport machinery.

Mutated NAC1 lacking nuclear localization ability cannot complement for loss of wild-type NAC1

NAC1 represses transcription of the candidate tumor suppressor GADD45GIP1 (6). To investigate the role of nuclear localization ability of NAC1 in the transcriptional regulator function of NAC1 in HeLa cells, we carried out RNAi complementation experiments. We expressed exogenous NAC1 in HeLa cells. The construct harbored six silent mutations in the siRNA-targeting region that render the corresponding NAC1 mRNA insensitive to the siRNA, denoted (si-R) for resistant hereinafter, but had no effect on the amino acid sequence of expressed NAC1 protein. Immunoblot analysis verified that these constructs were indeed resistant to knock down and that endogenous NAC1 could be efficiently eliminated from cells with NAC1-specific siRNA (Figure 6A and B). Using these stable cell lines, we asked whether the exogenously expressed proteins could complement for the loss of endogenous NAC1 by siRNA. As expected, siRNA-mediated knock down of endogenous NAC1 resulted in increased protein level of GADD45GIP1 (Figure 6A, lane 2). Introduction of a cDNA encoding only wild-type NAC1 (si-R) was sufficient to restore inhibitory function (Figure 6A, lanes 3 and 4). By contrast NAC1mut-R (si-R) lacking nuclear localization ability was not able to complement for loss of endogenous NAC1 expression (Figure 6A, lanes 5 and 6). These observations show that nuclear localization ability is essential for the transcriptional regulator function of NAC1 in cells.

Large molecules (>50kDa) cannot passively diffuse through nuclear pores (25), yet NAC1 with a theoretical molecular weight of 57.3 kDa must be imported into the nucleus through nuclear pores to function as a transcriptional regulator. This study showed that NAC1 harbors...
Fig. 5. Importin binding property of NAC1. (A–C) Importin binding was investigated by GST pull-down assays as described under Materials and methods. GST-tagged proteins were precipitated from reaction mixtures using glutathione-Sepharose 4B beads and importin-bound proteins were detected by Coomassie blue staining. (A) NAC1 (180–207) tagged with mC was subjected to pull-down with GST (lane 2) or GST-tagged importin α1, α3 and α6 (lanes 3–5). Lane 1 contains free (unbound) standard mC-tagged NAC1 (180–207) polypeptide. (B) NAC1 (180–207), mC and mC-tagged nucleoplasmin (NLS) were subjected to pull-down with indicated GST-tagged importin α3s (lanes 4–9). Lanes 1–3 contain standard mC, mC-tagged nucleoplasmin (NLS, 155–172) and free (unbound) NAC1 (180–207) polypeptide. (C) Mutated NAC1 polypeptides or mC-tagged NAC1 (180–207) were subjected to pull-down with GST-tagged importin α3 (lanes 6–10). Lanes 2–5 contain standard mC-tagged NAC1 (180–207) or mutated versions in the NLS of free (unbound) NAC1 polypeptide. (D) We expressed importin α3 or α4 fused with GST in 293T cells and purified using glutathione-Sepharose 4B beads and performed GST pull-down assays. Precipitated GST or GST-tagged proteins using glutathione-Sepharose 4B beads and importin-bound proteins were detected by Amido Black staining and anti-mC antibody, respectively. (E) Bipartite NLS of NAC1 binds to importin α. The amino-terminal armadillo repeat (Arm 3) and C-terminal repeat (Arm 8) comprising major and minor binding grooves are represented in pink and blue, respectively. Region containing conserved tryptophan and asparagine residue (Trp195 and Asn199 in human) essential for major binding site functions are marked. Three-dimensional image of yeast importin α (Protein Data Bank code 1EE5) was constructed using PyMOL.
a true functional NLS based on the following criteria. ‘Firstly’, NAC1 possesses a classical bipartite NLS sequence (KR at one end and RKXAK/R at the other) from different species (Figure 3A). The central linker region between two basic clusters usually consists of 10–12 amino acids (29). In this respect, the linkers of the NAC1 family (13–21 amino acids) are atypically long. However, their sequence contexts are glycine–serine rich (Figure 3A). Given that the NAC1 linker region is mutation-tolerant (29,30), its flexibility might allow significant movement and provide proper spacing between two positively charged clusters. ‘Secondly’, the sequence must be required for nuclear import. Full-length NAC1 or deletion mutants (2–250) were located in the nucleus (Figure 2C, panels C and D), whereas NAC1 transport into the nucleus was abolished when the sequence was deleted [deletion mutants (2–129 and 2–125)] (Figure 2C, panels E and F). ‘Thirdly’, the sequence must be sufficient to target an unrelated protein to the nucleus. We fused the sequence to the terminus of GFP and then visually assessed the location of the reporter protein. The NAC1 sequence (180–207) fused to GFP accumulated in the nucleus (Figure 3B). ‘Fourthly’, the sequence must directly interact with its putative import receptor importin α, a major carrier of cargo from the cytoplasm into the nucleus. To examine whether NAC1 conforms to this criterion, we examined direct binding in vitro using purified proteins. GST pull-down assays revealed that the sequence directly associated with importin α3/α4, but not with importins α1 or α6 (Figure 5A and 5D). Furthermore, replacing the basic residues in the sequence of NAC1 or mutations of the major NLS binding site of importin α3 eliminated the NAC1 and importin α3 binding capacity, thus clarifying that the sequence mediated the interaction.

The BTB domain comprises a stretch of ~95–125 amino acids and a protein–protein interaction motif that exists in about 183 genes in the human genome (31). The structure and sequential analysis predict that proteins containing a BTB domain have a variety of functions and participate in several cellular processes such as cytoskeleton dynamics, ion channel assembly and gating and transcriptional regulation. A large group of proteins in the family also contain C2H2 type zinc finger DNA-binding motifs and 43 BTB-ZF proteins have been identified in the human genome (31). Some BTB-ZF proteins are linked mainly to hematomafugal cancers (e.g. ZBTB16/PLZF (32), BCL-6 (33) and ZBTB32/FAZF (34)), and others are associated with solid tumors (e.g. Hic-1 (35), ZBTB7A/Pokemon (36), ZBTB7C/AMF-1 (37), GMCL1/GCL (38) and Kaiso (39)). The BTB-ZF protein Kaiso contains a classical monopartite NLS and it directly interacts with mouse importin α2 (note that mouse importin α2 is the orthologue of human importin α1) (40). The BTB-ZF protein Znf131 reportedly has two monopartite NLSs and it preferentially interacts with importin α3 (41). Although most known BTB proteins predominantly localize in the nucleus, the mechanisms of this process remain largely unknown. We could not identify the NAC1 NLS by a series of in silico searches using such tools as PROSITE or MotifScan and thus conducted a visual examination. One of the authors (SK) recently developed a highly accurate NLS prediction program (cNLS Mapper) that calculates NLS activity scores using activity-based, but not sequence-based, profiles of importin α-dependent NLS (42). Analysis of human NAC1 using this software provided the correct information, whereas the other NLS prediction programs, PSORT II (http://psort.hgc.jp/form2.html) and PredictNLS (http://cubic.bioc. columbia.edu/cgi/var/nair/resonline.pl), failed to predict the NAC1 NLS. Thus, cNLS Mapper is an informative tool for predicting classical NLS, and it is freely available at http://nls-mapper.iab.keio. ac.jp/cgi-bin/NLS_Mapper_form.cgi. The identification of NLS with nuclear proteins such as the transcriptional repressors is a key step to delineate the molecular dynamics and plasticity underlying various cellular processes. We anticipate that this powerful prediction tool will be useful for laying the foundation for understanding the transcriptional regulation and networks of proteins containing a BTB domain with respect to oncogenic properties.

The BTB domain mediates homodimerization as well as protein–protein interactions, allowing the recruitment of co-repressor complexes (9,12). In this respect, studies of functional NAC1 inhibition using a truncated NAC1 with potential dominant-negative activity on NAC1 provided important clues to understanding its putative oncogenic role (1,6,7). Here, we introduced point mutations into the BTB domain using structural information (12) and developed a Y86A mutant of NAC1 with resistance to dimer formation, and found that dimer formation is not committed to the nuclear localization of NAC1 in the presence of the NLS sequence. The point mutant will be a useful tool with which to study how dimer formation contributes to transcriptional regulation and protein–protein interactions associated with oncogenic properties.

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**Fig. 6.** Expression of wild-type, but not mutated NAC1 lacking nuclear localization ability inhibited NAC1-siRNA-induced expression of GADD45GIP1. (A) Immunoblot showing levels of NAC1 and GADD45GIP1 in cells. The siRNA-treated cells were solubilized and equal volumes loaded onto a 12% polyacrylamide gel. GADD45GIP1 was detected by immunoblotting using a mouse polyclonal antibody. (B) Protein expression level of GADD45GIP1 in NAC1-siRNA-treated cells. The values presented are the mean ± S.D. from three independent experiments normalized to the levels in control siRNA-treated cells.
In contrast, a dimerization-dependent NLS of signal transducers and activators of transcription 1 protein has been constructed (43).

In summary, we developed a monoclonal antibody against NAC1. Analyses of cell lines and tissues confirmed that NAC1 is a nuclear protein and that dimer formation is not committed to the nuclear localization of NAC1. We identified a novel site on NAC1 that is conserved across species. This region is sufficient to direct the nuclear import of GFP as it binds importin α3/α4, and mutations in this region abolished such binding. These results indicated that the 183–205 basic region of human NAC1 is a functional NLS. We plan to define the NAC1 consensus DNA-binding sequence for the genome-wide study of NAC1-regulated genes in cells, and to investigate whether its downstream target genes are repressed or activated, and how NAC1 proteins function in the nucleus of cancer cells.

Supplementary material

Supplementary materials can be found at http://carcin.oxfordjournals.org/.

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