Analysis of the Signals and Mechanisms Mediating Nuclear Trafficking of GATA-4

LOSS OF DNA BINDING IS ASSOCIATED WITH LOCALIZATION IN INTRANUCLEAR SPECKLES*

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Nucleocytoplasmic transport of GATA-4 is important in maintaining and regulating normal cardiogenesis and heart function. This report investigates the detailed mechanisms of GATA-4 nuclear transport. We characterized a nonclassical nuclear localization signal between amino acids 270 and 324 that actively transports GATA-4 into the nucleus of both HeLa cells and cardiac myocytes. Fine mapping studies revealed four crucial arginine residues within this region that mediate active transport predominately through the nonclassical pathway via interaction with importin β. These four residues were also essential for the DNA binding activity of GATA-4 and transcriptional activation of cardiac-specific genes. Interestingly, mutation of these residues not only inhibited DNA binding and gene transcription but also resulted in a preferential accumulation of the GATA-4 protein in distinct subnuclear speckles. A cardiac myocyte-specific, chromosome maintenance region 1-dependent nuclear export signal consisting of three essential leucine residues was also identified. The current study provides detailed information on the nuclear shuttling pathways of GATA-4 that represents an additional mechanism of gene regulation.

The heart is the first organ to form during embryogenesis. It arises through a complex process that requires cooperative interactions between various cytokines and specific transcription factors such as GATA-4 (1). GATA-4 is a cardiac-specific member of the GATA family of zinc finger transcription factors (2). Members of this family contain two conserved type IV zinc finger motifs of the distinctive form Cys-Xaa2-Cys-Xaa17-Cys-Xaa2-Cys (3). These fingers recognize and bind a consensus sequence known as the “GATA” motif (A/T(GATA)A/G), present in the transcriptional regulatory regions of a plethora of cardiac-specific genes (4). GATA-4 is considered one of the earliest markers of cardiac myocyte commitment and has been shown to be essential for normal heart formation, with GATA-4 knockout mice dying between 8.5 and 10.5 days postcoitum and displaying profound abnormalities in ventral morphogenesis and heart tube formation (5). Furthermore, RNA-inducible inhibition of GATA-4 in the P19 embryonic stem cell line showed diminished activation of cardiac-specific genes and a resulting decrease in expression of cardiac muscle markers and cardiac myocyte differentiation (6). In contrast, ectopic expression of GATA-4 in P19 cells led to a 10-fold increase in the number of terminally differentiated beating cardiac myocytes (7). Moreover, overexpression of GATA-4 can also have detrimental effects on the developed heart such as induction of cardiac hypertrophy (8). Hence, considering the need for precise regulation of GATA-4 in both heart development and normal cardiac function, it is essential that we define the molecular pathways regulating GATA-4 activity, one of which is nuclear shuttling.

Like all nuclear proteins, GATA-4 is synthesized in the cytoplasm and must be imported into the nucleus to perform its transcriptional function. This nucleocytoplasmic transport across the nuclear envelope occurs via the nuclear pore complex (NPC)9 (9). Small molecules can passively diffuse through the NPC, whereas larger molecules are actively transported in and out of the nucleus. This active transport process requires nucleocytoplasmic shuttling proteins known as importins and exportins (10) that recognize nuclear localization signals (NLS) (11, 12) and nuclear export signals (NES) (13), respectively. NLSs are sequences that are necessary and sufficient to allow a protein to be targeted to the nucleus. Although there is not a strict consensus sequence, classical NLSs are typically small stretches of positively charged amino acids, arranged as either monopartite (a single cluster) or bipartite (two clusters separated by a 10–12-amino acid spacer) sequences (14, 15). NESs, however, allow a protein to be exported from the nucleus and are characteristically short stretches of leucine-rich and hydrophobic amino acids (16). The import of substrates with a classical NLS is mediated by the classical pathway whereby impor-
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...alpha binds the NLS-containing protein in the cytoplasm. Importin beta then binds to importin alpha, forming a trimeric complex. This complex is then translocated through the NPC by transient importin beta-mediated interactions with nucleoporins. Once in the nucleus, RanGTP binds to importin beta, dissociating it from importin alpha and the NLS-containing protein. Importin alpha then dissociates from the NLS cargo in the presence of the export receptor cellular apoptosis susceptibility gene (CASS). The importins are then recycled back to the cytoplasm for another round of transport, leaving the NLS-containing protein in the nucleus to perform its function.

Although certain classes of proteins are imported via this classical pathway, studies have identified several types of non-classical pathways that do not require importin alpha for NLS recognition. In this case, the NLS of the target protein can either interact directly with the nucleoporins of the NPC or interact with one of the many homologues of importin beta for translocation into the nucleus.

Although nucleocytoplasmic transport is an important regulator in the biological activity of GATA-4 as a transcription factor, it is unknown which amino acid residues of GATA-4 serve as signals for its nucleocytoplasmic transport and which pathways it utilizes. In this study we elucidated that GATA-4 contains a cardiac-specific, CRM1-dependent NES, with three leucine residues essential for export. We also identified a functional NLS in the DNA-binding domain (DBD). This NLS facilitates nuclear import predominately via the nonclassical pathway through interaction with importin beta and RanGTP. Mutational analyses led to the identification of a group of four positively charged residues that are crucial for nuclear import, forming a platform for interaction with importin beta. We also identified the DNA-binding residues involved in the activity of GATA-4. Mutation of these residues not only abolished DNA binding activity and transcriptional activation of cardiac-specific genes but surprisingly was also associated with an intranuclear accumulation of the mutant GATA-4 protein in speckles. Furthermore, we demonstrated that DNA molecules containing GATA-binding sites and the presence of RanGTP effectively compete with importin beta for binding the GATA-4 DBD, suggesting that DNA binding and RanGTP play a role in the release of importins for recycling back to the cytoplasm.

EXPERIMENTAL PROCEDURES

Plasmids—Mouse full-length GATA-4 (accession number NM_008092) and deletion fragments were subcloned into: pDEST53 and pDEST47 vectors for N- and C-terminal GFP fusion, respectively; pDEST15 vector for C-terminal GST fusion; and pAd/CMV/V5-DEST vector for adenoviral gene delivery (Gateway Technology; Invitrogen). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The brain natriuretic peptide (BNP) promoter region was cloned upstream of the luciferase reporter in the pGL3Basic vector (Promega, Madison, WI). The importin alpha (PTAC58) subunit and importin beta (PTAC97) subunit cloned into the pGEX2T vector were kindly donated by Yoshihiro Yoneda (Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, Osaka, Japan). Nup62 in the pDNA3.1/HisB vector and Nup153 in the pET28b vector were generous gifts from Professor N. Yaseen (Feinberg School of Medicine, Northwestern University, Chicago, IL).

Cell Culture—Human embryonal kidney 293A cells were obtained from Invitrogen. Human HeLa cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Primary cultures of beating neonatal myocardial cells were isolated from 2–3-day-old rats as previously described (57, 58). Briefly, the rat hearts were isolated and digested in the presence of 0.05% collagenase type II (Worthington Biochemicals, Freehold NJ). Differential plating was performed to purify cardiac myocytes from other cell types such as fibroblasts. The cells were then subjected to red blood cell lysis. 1 x 10^5 cells were plated onto collagen-coated coverslips (BD Biosciences, Sydney, Australia). All of the cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% dialyzed fetal bovine serum (Invitrogen) and maintained at 37 °C in a humidified atmosphere of 5% CO2, 95% air. 293A cells were also supplemented with 4.5 g/liter of d-glucose (Sigma).

Transient Cell Transfection—HeLa cells were grown on coverslips at 2 x 10^5 cells/well in 6-well plates. The following day the cells were transiently transfected with 1.2–2 μg of GATA-4-GFP expression vectors using Lipofectamine or Lipofectamine 2000 as per the manufacturer’s instructions (Invitrogen). The cells were fixed with 4% paraformaldehyde 48 h after transfection and analyzed with an Olympus confocal microscope (Olympus, Tokyo, Japan) at 600× magnification. The images were acquired using Olympus Fluoview software, version 4.3, FV300 (Olympus Optical Co. Ltd.).

Adenoviral Transduction and Antibody Staining—Viral supernatant was produced using 293A cells as per the manufacturer’s instructions (Invitrogen). Cardiac myocytes were serum-starved for 24 h and transduced for a further 24 h with an equal number of viral particles (multiplicity of infection of 25) derived from the pAd-LacZ or pAd-GATA-4 constructs. Viral supernatant was replaced with fresh serum-free medium, and the cells were cultured for 24 h. The cells were then fixed in 100% methanol. Following blocking with 5% skim milk in phosphate-buffered saline, the cells were stained with anti-alpha-actinin monoclonal antibody (A7811; Sigma; 1:800 dilution) for 45 min. The secondary antibody, Alexa Fluor 594 (A-11020; Invitrogen; 1:500 dilution) was applied for 45 min. This was followed by the addition of an anti-V5-fluorescein isothiocyanate monoclonal antibody (R963-25; Invitrogen; 1:500 dilution) for 45 min. After each antibody incubation the slides were washed three times with phosphate-buffered saline. The coverslips were mounted onto the slides using glycerol (Sigma), and the cells were viewed with an Olympus confocal microscope. For nuclear export studies, the transduced cells were treated with 7.5 ng/ml leptomycin B (LMB) for 5 h prior to fixing.

GST Pull-down Assay—GST fusion proteins were expressed in Escherichia coli strain BL21 (DE3) (Promega) as formerly described (59). 35S-Labeled GATA-4, deletion constructs, and mutants were in vitro translated using the TNT T7 quick coupled transcription/translation system (Promega) as per the manufacturer’s instructions. GST pull-down assays were performed as previously detailed (36). For Ran studies (see Fig. 5B),
0.9 μM RanGDP or RanGTP (Ran Q69L, a dominant GTP form of Ran; Calbiochem, La Jolla, CA) was added. For DNA competition studies (see Fig. 10), 200 ng of BNP probe or scrambled BNP probe was added.

siRNA Transfection—Small interfering RNA molecules (siRNAs) corresponding to exon 3 and exon 9 of human importin β (catalogue number 16707, siRNA identification codes 11125 and 145041, respectively) were obtained from Ambion (Austin, TX). A negative control no. 1 (catalogue number 4611) was also purchased.

Transfection of siRNA was performed using siPORT NeoFX (Ambion) as per the manufacturer’s instructions. Briefly, 2.3 × 10⁵ HeLa cells were transfected with 30 nM siRNA and 5 μl of NeoFX reagent for 48 h. The cells were subsequently transfected with the GATA-4-GFP expression vector using Lipofectamine 2000, as previously described. After 24 h, the cells were harvested, and nuclear localization of GATA-4 was examined using confocal microscopy.

Western blot analyses were performed to assess importin β knockdown 48 and 72 h after siRNA transfection using an anti-importin β antibody (sc-1863; Santa Cruz, CA; 1:200 dilution). Equal protein loading was measured by Ponceau S staining.

Luciferase Assay and Western Blot Analysis—HeLa cells were seeded at 2 × 10⁵ cells/well in 6-well plates. The following day the cells were transiently transfected with 200 ng of the BNP reporter plasmid together with 800 ng of wild type or mutant GATA-4 expression vectors using Lipofectamine according to the manufacturer’s instructions (Invitrogen). After 48 h, the cells were harvested, and luciferase activity was assessed using the dual luciferase assay kit (Promega) as per the manufacturer’s protocol. Briefly, the cells were washed with phosphate-buffered saline and lysed with 0.5× passive lysis buffer (Promega) containing complete protease inhibitor (Roche Applied Science). Luciferase activity was measured using the TD-20/20 luminometer (Turner Designs). To confirm that GATA-4 protein was expressed in transiently transfected cells, Western blot analysis was carried out as previously described (57). The membranes were probed using a goat anti-GATA-4 antibody (sc-1237; Santa Cruz; 1:500 dilution), followed by an anti-goat antibody conjugated with horseradish peroxidase (P 0449; DAKO, Denmark; 1:5000 dilution). Protein was detected using Western Lightning Chemiluminescence reagent plus (PerkinElmer Life Sciences) in conjunction with exposure to CL-Xposure Film (Quantum Scientific, murarrie, QLD, Australia).

Electromobility Shift Assay (EMSA)—The double-stranded DNA probe (ATG CAG CTG ATA AAT CAG AGA) containing a GATA-binding site (in bold type) was selected from the promoter of mouse BNP and end-labeled with [³²P]dATP (Amersham Biosciences) by polynucleotide enzyme as previously described (36). Wild type GATA-4 and its mutants were in vitro translated using the TnT T7 quick coupled transcription/translation system (Promega) and allowed to bind to the mBNP probe (2 × 10⁵ cpm) for 30 min. The samples were then incubated for 30 min with a goat anti-GATA-4 antibody (sc-1237; Santa Cruz; 1:50 dilution) for supershift studies. The samples were loaded onto a non-denaturing 5% polyacrylamide gel, electrophoresed, and dried, and autoradiography was performed. To ensure equal expression of the in vitro translated protein, parallel experiments were performed with [³⁵S]methionine. These samples were boiled in the presence of 20% β-mercaptoethanol, electrophoresed on a 10% SDS-polyacrylamide gel, dried, and exposed to Kodak BioMax MR-1 x-ray film (GE Healthcare Bioscience).

RESULTS

Classification of the GATA-4 NLS—The GATA-4 DBD consists of two zinc finger motifs and two adjacent stretches of basic amino acids (aa 216–324) (24) (UniProtKB/Swiss-Prot entry Q08369) (Fig. 1A). Furthermore, it contains three transcriptional activation domains: two N-terminal independent domains (aa 1–74 and 130–177) and one C-terminal domain that is required for transcriptional activity of the native protein but is insufficient to independently activate transcription (25) (Fig. 1A).

To identify the region of GATA-4 that contains an NLS and is therefore responsible for its nuclear import, full-length GATA-4 and various GATA-4 deletion fragments were fused upstream or downstream of GFP (Fig. 1B). GFP alone (27 kDa) does not contain an NLS and can travel freely between the nucleus and the cytoplasm (26, 27). Hence, a GFP construct was used as a negative control. These constructs were transiently transfected into HeLa cells, and the intracellular localization of the fusion proteins was analyzed by confocal microscopy (Fig. 1C).

As shown in Fig. 1C, GFP alone (Mock) is evenly distributed in the nucleus and cytoplasm, indicating the absence of an active NLS. Full-length GATA-4 fused to GFP at either the C or N terminus (Fig. 1B, panels i and ii) were expressed and actively targeted to the nucleus (Fig. 1C, panels i and ii), suggesting the presence of an intact and functional NLS. Removal of the C-terminal activation domain (Fig. 1B, panel iii), of the N-terminal activation domains (Fig. 1B, panel vi), or of all activation domains (Fig. 1B, panel vii) had no effect on nuclear localization, with all three GATA-4-GFP fragments being actively targeted to the nucleus (Fig. 1C, panels iii, vi, and vii). Furthermore, the N- and C-terminal fragments of GATA-4 (Fig. 1B, panels iv and v) were insufficient to direct nuclear accumulation independently (Fig. 1C, panels iv and v). Indeed, of the deletion constructs generated, only those that contain the C-terminal zinc finger and adjacent basic domain (aa 270–324) (Fig. 1B, panels iii, vi, vii, ix, and x) were found to be targeted to the nucleus, similar to full-length GATA-4 (Fig. 1C, panels iii, vi, vii, ix, and x). Therefore, the minimal domain that contains an intact NLS and is thus responsible for active nuclear import of GATA-4 was determined to be from amino acids 270–324 (Fig. 1B, panel xi). To further confirm this finding, deletion of this region in the full-length GATA-4 (Fig. 1B, panel xii) showed complete loss of actively transported protein to the nucleus (Fig. 1C, panel xii).

From these experiments, it can be ascertained that the C-terminal zinc finger and adjacent basic domain (aa 270–324) is sufficient for complete nuclear localization of GATA-4. Unexpectedly, those GATA-4 constructs that did not contain an NLS were detected in both the nucleus and the cytoplasm. Because none of the fragments were excluded from the nucleus, it sug-
gested that either GATA-4 does not contain an NES or the NES is not functional in HeLa cells.

To determine whether the zinc finger structure of GATA-4 was involved in its nuclear import, GATA-4 C270A/H11001C273A was produced by mutation of the cysteine residues to alanine, thus effectively destroying the C-terminal zinc finger of GATA-4. This had no effect on the ability of GATA-4 to be targeted to the nucleus (data not shown).

Individual Mutations of the Positively Charged Amino Acids in the GATA-4 NLS Are Insufficient to Inhibit Nuclear Localization—To determine the specific amino acid residues necessary for the nuclear import of GATA-4, point mutations were introduced into the full-length GATA-4 to change the positively charged residues, arginine and lysine, typically involved in NLS, into the neutral amino acid alanine (Fig. 2A).

The following mutants in GATA-4 were generated: R282A, R283A, K299A, R305A, R310A, K311A, R317A, K318A, R319A, K320A, and K322A. These GFP fusion constructs were transiently transfected into HeLa cells, and their intracellular location were analyzed by confocal microscopy (Fig. 2B).

Of the 11 single point mutant constructs transfected, none had the ability to inhibit nuclear targeting of GATA-4-GFP, as observed by exclusive localization of GFP in the nucleus (Fig. 2B). Interestingly, it was noted that several of the mutant proteins, in particular R282A, R283A, K299A, R305A, and R317A, accumulated preferentially in subnuclear speckles (Fig. 2, B and C).

Multiple Mutations Are Required to Inhibit Nuclear Import of GATA-4—Because single mutations had no effect on nuclear localization of GATA-4, the NLS region was examined for clusters of positively charged amino acids. Three clusters (similar to classical NLS clusters) were identified: cluster 1 consisting of two adjacent residues, Arg282 and Arg283; cluster 2 also consisting of two adjacent residues, Arg310 and Lys311; and cluster 3 consisting of five residues, Arg317, Arg318, Arg319, Arg320, and Arg322. Multiple mutations were made, and nuclear localization was examined (Fig. 3).

Mutation of each of the individual clusters was insufficient to inhibit nuclear targeting of GATA-4, with the protein still accumulating in the nucleus of HeLa cells (Fig. 3, panels i–iii). Likewise, simultaneously mutating both clusters 2 and 3, and clusters 1 and 2 had no effect on GATA-4 nuclear localization (Fig. 3, panel iv, and data not shown). In contrast, mutating both clusters 1 and 3 simultaneously abolished nuclear import, resulting in diffuse staining throughout the nucleus and the...
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Intracellular Localization of GATA-4 Constructs and Mutants in Cardiac Myocytes—The above nuclear localization experiments were carried out in HeLa cells, because of their high transfection efficiency and absence of endogenous GATA-4 expression (28). However, because GATA-4 is involved in cardiac development and function, a more biologically relevant cell type was used to verify our results, namely cardiac myocytes. In Fig. 4, rat cardiac myocytes were transduced with V5-tagged GATA-4 wild type, fragments, and mutants, and their nucleocytoplasmic distribution were examined. The localization of the V5-tagged GATA-4 constructs was determined using an anti-V5-fluorescein isothiocyanate antibody, and cardiac myocytes were stained with an anti-α-actinin antibody.

The small, diffusible reporter protein LacZ used as our negative control accumulated in large spots throughout the cell (Fig. 4A, panel i). Similar to the results observed in HeLa cells (Figs. 1C and 3), full-length GATA-4, the minimal NLS region (aa 270–324) and simultaneous mutation of clusters 2 and 3, were all targeted to the nucleus of cardiac myocytes (Fig. 4A, panels ii, iii, and vi). However, whereas FL ΔNLS and the mutant that abolished nuclear accumulation (FL R282A + R283A + R317A + R319A) were no longer targeted to the nucleus as expected, the protein was exclusively localized to the cytoplasm (Fig. 4A, panels iv and v). This was in contrast to the results observed in HeLa cells where the protein was distributed evenly throughout the nucleus and the cytoplasm (Figs. 1C and 3). These results suggested that either these fragments were simply not transported into the nucleus or GATA-4 contains an NES that is operational in cardiac myocytes.

CRM1 Is the Export Receptor for the GATA-4 NES—To further investigate the possibility of a functional NES in cardiac myocytes, the cells were transduced with various GATA-4-V5 fragments in the presence of the CRM1 nuclear export inhibitor, LMB. Their nucleocytoplasmic distribution was examined (Fig. 4B).

The presence of LMB had no effect on LacZ or the constructs that had a functional NLS (Fig. 4B, panels ii, iii, and vi). However, after treatment with LMB, FL ΔNLS (Fig. 4B, panel iv) and the mutant that abolished nuclear import (FL R282A + R283A + R317A + R319A) (Fig. 4B, panel v) were detected in both the nucleus and the cytoplasm, suggesting that nuclear export had in fact been inhibited by LMB. It is likely that the GATA-4 constructs that lack a functional NLS can indeed still passively diffuse into the nucleus in both HeLa cells and cardiac myocytes. However, the presence of a functional NES in cardiac myocytes may explain the cytoplasmic-restricted localization of the protein in these cells.

Identification of a Leucine-rich Nuclear Export Signal in GATA-4—To ascertain the specific amino acids necessary for GATA-4 export, we examined the GATA-4 sequence and identified a region in the N-terminal activation domain that contains three leucine residues resembling a typical NES consensus. These three leucine residues were simultaneously mutated to alanine in FL ΔNLS. Rat cardiac myocytes were transduced with this V5-tagged GATA-4 mutant, and its localization was determined.

cytoplasm (Fig. 3, panel v). To further fine map the exact amino acids involved, many combinations of the seven mutants in clusters 1 and 3 were generated (Fig. 3, panels vii and vii, and data not shown for other mutants). Simultaneous mutation of one amino acid from both clusters 1 and 3 was inadequate to abolish nuclear localization of GATA-4 (Fig. 3, panel vi). However, only simultaneous mutation of R282A + R283A (from cluster 1), together with mutation of R317A + R319A (from cluster 3) was sufficient for complete inhibition of GATA-4 nuclear import (Fig. 3, panels vii). Hence, amino acids Arg282, Arg283, Arg317, and Arg319 are crucial for nuclear localization of GATA-4.
As can be observed in Fig. 4 (panel vii), the NES mutant is no longer cytoplasmic-restricted and is now distributed throughout the nucleus and cytoplasm. The presence of LMB had no effect (Fig. 4 B, panel vii). Hence, amino acids Leu49, Leu51, and Leu54 are crucial for the nuclear export of GATA-4.

**Nuclear Import Mechanisms of GATA-4**—Three common mechanisms have been reported for active nuclear import of proteins containing an NLS, that is: (i) the classical pathway, mediated by both importin α and importin β; (ii) the nonclassical pathway where recognition and import is carried out by importin β alone; or (iii) via direct interactions with the nucleoporins of the NPC. To determine which pathway GATA-4 utilizes for its nuclear import, GST pull-down assays were performed.

For the classical and nonclassical import studies, 35S-GATA-4 constructs and mutants were allowed to interact with the GST fusion proteins, importin α, and importin β or with GST alone as a negative control.

FL GATA-4 and the minimal NLS region (aa 270–324) did not interact with importin α but bound strongly to importin β (Fig. 5A, panels i and ii, lanes 2 and 3). In contrast, FL ΔNLS, was unable to bind either importin α or importin β (Fig. 5A, panels iii, lanes 2 and 3). This suggested that GATA-4 import is mediated via the nonclassical pathway through interaction between importin β and the NLS region.

To further elucidate the role of the amino acids found to be involved in nuclear import of GATA-4, the interaction of 35S-GATA-4 mutants with importin β was investigated. FL R282A + R283A + R317A + R319A completely abolished importin β binding (Fig. 5A, panels iv, lane 3). In contrast, simultaneous mutation of clusters 2 and 3 only slightly reduced importin β binding (Fig. 5A, panel v, lane 3). This correlates with the nuclear localization data observed for these mutants (Figs. 3 and 4) and indicates that amino acids Arg282, Arg283, Arg317, and Arg319 mediate nuclear import by interaction with importin β.

To determine whether the import of GATA-4 is also nucleoporin-mediated, GST pull-down studies were carried out. 35S-Nup62 and -Nup153 were allowed to interact with the GST fusion protein GATA-4 or with GST alone as a negative control. These studies showed that GATA-4 consistently interacts with and binds nucleoporins, albeit weakly (data not shown).

Taken together, it appears that GATA-4 can be imported by both the nonclassical nuclear import pathway and via direct interactions with nucleoporins. The preferential mechanism appears to be the nonclassical pathway that requires the interaction of importin β with amino acid residues Arg282, Arg283, Arg317, and Arg319.

Once the NLS-importin β complex has transversed the NPC, dissociation of the complex is usually mediated by binding of RanGTP to importin β (29). Therefore, to ascertain whether Ran plays a role in the dissociation of GATA-4 and importin β, GST pull-down studies with 35S-GATA-4 and GST-importin β were carried out in the presence of the active and inactive forms of Ran, that is, RanGTP and RanGDP, respectively. As shown in the GST fusion proteins, importin α, and importin β or with GST alone as a negative control.
Fig. 5B, GATA-4 interacts strongly with importin β (Lane 2). RanGDP had no effect on the binding of importin β to GATA-4 (Fig. 5B, lane 3). In contrast, the addition of RanGTP dissociated the binding of importin β to GATA-4 (Fig. 5B, lane 4). These results indicated that the presence of RanGTP separates the GATA-4 NLS-importin β complex, suggesting a mechanism for importin release and recycling once GATA-4 has entered the nucleus.

Effect of Importin β Knockdown on GATA-4 Nuclear Localization—To further determine the role of the nonclassical pathway in GATA-4 nuclear import, importin β expression was selectively knocked down using siRNA targeted to exon 3 or exon 9 of importin β. After 48 h, the cells were transfected with GFP-tagged GATA-4 for a further 24 h, and nuclear localization was examined.

After 48 h, siRNA targeted to exon 3 and exon 9 of importin β had significantly decreased importin β expression by 54 and 78%, respectively, compared with control lysates (Fig. 6A). This suppressed expression continued up to 72 h (Fig. 6A). As expected, the negative control siRNA had no effect on importin β expression compared with control lysates (Fig. 6A).

Interestingly, despite significantly depressed importin β expression, GATA-4 was still actively targeted to the nucleus (Fig. 6B). This was surprising, because our previous experiments demonstrated an importin β-mediated import mechanism. However, because GATA-4 is a crucial transcription factor, it cannot be ruled out that in circumstances where the nonclassical pathway is inactivated, an alternate rescue mechanism becomes the dominant pathway to maintain normal GATA-4 nuclear import. Because we have demonstrated a role for nucleoporins in GATA-4 nuclear import, it is possible that this may be one such rescue pathway.

Accumulation of GATA-4 Mutants in Subnuclear Compartments Is Correlated to Loss of DNA Binding Activity—Individual mutations of positively charged residues within the NLS were insufficient to abolish nuclear localization, with the mutant GATA-4 protein still localized to the nucleus (Fig. 2B). However, unexpectedly, a number of mutants were found to aggregate solely within distinct subnuclear compartments,
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resulting in a speckled appearance (Fig. 2, B and C). Mutants with the highest percentage of cells containing speckles were R282A, R283A, R305A, and R317A, with 60, 27, 50, and 25% of the cells displaying accumulation of GATA-4-GFP in subnuclear speckles, respectively (Fig. 7). These residues are located in the overlapping NLS and DBD of GATA-4, and although individually mutating these residues did not affect nuclear localization (Fig. 2B), their role in DNA binding has not yet been examined.

To determine whether these residues affect the DNA binding activity of GATA-4, EMSAs were performed using a BNP promoter probe containing a GATA-binding site. All of the samples were supershifted using an anti-GATA-4 antibody to enable clearer visualization of the protein-DNA complex (Fig. 8A).

As expected, wild type GATA-4 bound the probe strongly (30) (Fig. 8, A and B, lane 3). However, seven of the 11 NLS residues were essential for DNA binding activity. A complete loss of DNA binding was observed for the mutants R282A, R283A, K299A, R305A, K311A, R317A, and R319A (Fig. 8, A and B, lanes 4–7, 9, 10, and 12). A reduction in DNA binding was observed for mutation of amino acids Arg310, Lys318, Lys320, and Lys322 compared with wild type (Fig. 8, A and B, cf. lane 3 with lanes 8, 11, 13, and 14). Indeed, there was a direct correlation between speckling and DNA binding activity (Fig. 8C). Mutants that demonstrated a higher occurrence of speckling were unable to bind DNA, whereas mutants that showed a lesser degree of speckling were able to bind DNA, albeit weakly (Fig. 8C).

The above results suggested that the four amino acids that when mutated simultaneously abolished nuclear localization, Arg282, Arg283, Arg317, and Arg319 (Fig. 3, panel vii), are also crucial for DNA binding. Thus, they have a dual function in regulating both nuclear import and DNA binding activity and, as such, are vital for GATA-4 function.

Interestingly, for the first time, we identified a direct correlation between loss of DNA binding activity and accumulation of a protein in distinct subnuclear speckles. This may represent targeting of transcription factors that are no longer able to bind DNA to specific intranuclear compartments. However, preliminary studies suggested that the mutant proteins were not colocalized to proteasomes, splicing factor compartments, or nucleolin (data not shown). To our knowledge, this is the first time a correlation between DNA binding activity and subnuclear localization has been documented, and further experiments are underway to study this phenomenon.

Transcriptional Activity of GATA-4 and Its Mutants—It has been shown previously that binding of GATA-4 to the cardiac-specific promoter of BNP is essential for gene transcription (31). To determine whether mutation of the positively charged residues not only affects nuclear import and DNA binding but also functional activity of GATA-4, a BNP promoter luciferase reporter was cotransfected with GATA-4 cDNA (wild type and its mutants) into HeLa cells, and luciferase activity assessed (Fig. 9). To demonstrate that all the GATA-4 constructs were translated into proteins, Western blot analyses of transfected HeLa cells was performed (Fig. 9, inset).

As previously reported (31), wild type GATA-4 notably activated the reporter (Fig. 9). All mutants showed a significant reduction in the activation of the BNP reporter compared with wild type. As expected, those mutants unable to bind DNA, i.e. R282A, R283A, K299A, R305A, K311A, R317A, and R319A, also failed to activate the BNP reporter compared with wild type (Fig. 9). Mutants R310A, K318A, K320A, and K322A, which showed reduced DNA binding activity, also demonstrated decreased luciferase activity compared with wild type (Fig. 9).

These results demonstrated that residues crucial for DNA binding are consequently also essential for transcriptional activity of GATA-4. Mutation of these residues results in the protein being localized to subnuclear speckles, unable to bind DNA and unable to activate transcription.

DNA Containing GATA-binding Sites Compete with Importin β for Binding the GATA-4 DBD—Our results indicated that the DBD spanning amino acids 216–324 also encompasses the NLS (aa 270–324) and therefore has a dual function in both DNA binding and nuclear import. Moreover, amino acids shown to be crucial for nuclear import are also important for
FIGURE 8. DNA binding activity of GATA-4 and its mutants. Wild type (Wt and WT) GATA-4 and its mutants in the pDEST47 vector were in vitro translated (inset of B). A, EMSA was carried out using a 32P-labeled probe containing a GATA-binding site. Supershift was performed using an anti-GATA-4 antibody. B, densitometry of EMSA data. Typical experiment of three performed. C, correlation between DNA binding activity and subnuclear speckles.
proteins were expressed in transfected cells (**, relative to a value of 1. The data are representative of three experiments. The results, normalized to mock vector, are expressed as the means ± S.D. relative to a value of 1. **, p < 0.01. Western blot analyses were performed to ensure that all GATA-4 proteins were expressed in transfected cells (inset), WT, wild type.

binding DNA. However, whether this region can be occupied by both GATA-specific DNA and importin β simultaneously or whether there is competition for occupancy is yet to be elucidated. To investigate this, we determined the influence of DNA containing a GATA-binding site (BNP promoter probe) and nonspecific DNA (scrambled BNP promoter probe) on the GATA-4/importin β interaction using the GST pull-down assay.

In vitro translated 35S-GATA-4 was allowed to interact with GST-importin β alone or in the presence of specific DNA or nonspecific scrambled DNA (Fig. 10, lanes 2–4, respectively). As previously shown in Fig. 5, GATA-4 strongly interacted with importin β (Fig. 10, lane 2). However, the presence of specific BNP promoter DNA significantly reduced the ability of GATA-4 to interact with importin β (Fig. 10, lane 3). In contrast, presence of the nonspecific scrambled BNP DNA had no effect on the interaction between GATA-4 and importin β (Fig. 10, lane 4).

These results showed that in the presence of BNP promoter probe, importin β binding to GATA-4 is greatly reduced. This suggested that importin β and GATA-specific DNA have overlapping recognition sites within the NLS/DBD region and cannot effectively occupy this region simultaneously.

DISCUSSION

GATA-4 is a transcription factor that plays an important role in the development and function of the vertebrate heart (32). Absence of GATA-4 has been implicated in congenital heart disease (5), and overexpression of GATA-4 is involved in cardiac hypertrophy (8). Thus, precise regulation of GATA-4 transport in and out of the nucleus, where gene transcription occurs, is paramount.

In the current study, we identified the minimal NLS region (aa 270–324) that can actively transport GATA-4 into the nucleus of both HEK cells and cardiac myocytes. This is in agreement with a previous study by Morrisey et al. (25), who identified the NLS to a larger fragment encompassing aa 251–324 using NIH3T3 cells. We further characterized that this active transport process is mediated predominately through the nonclassical pathway via interaction with importin β. Interaction with nucleoporins 62 and 153 was also detected in vitro. Fine mapping studies revealed four arginine residues (Arg282, Arg283, Arg317, and Arg319) that are crucial for importin β-mediated nuclear import. In addition, these four crucial arginine residues are also essential for DNA binding activity and hence transcriptional activity of GATA-4. We also discovered a cardiac myocyte-specific, CRM1-dependent NES in GATA-4 that consists of three indispensable leucine residues.

The four crucial residues in the NLS of GATA-4 are arranged in two clusters separated by a 33-amino acid spacer (Fig. 2A). The location of these four crucial amino acids, Arg282, Arg283, Arg317, and Arg319, does not conform to the “classical” monopartite or bipartite sequence, which is typically recognized by importin α (14, 15, 17). Moreover, the GATA-4 NLS consists of only arginine residues and is imported by importin β (Fig. 5A), which is typical of nonclassical NLSs (ncNLSs) (23). Therefore, GATA-4 represents a ncNLS.

Interestingly, GATA-4 belongs to a family of transcription factors that share a highly conserved DNA-binding domain. Indeed, within this region the cardiac-specific GATA-4, -5, and -6 share ~85% homology, and mouse GATA-4 is ~70% identical to both mouse and Drosophila pannier GATA-1 (24). Fig. 11A depicts the consensus amino acid sequence of the minimal NLS region of GATA-4 within this DBD. The four arginine residues vital for GATA-4 nuclear import are shown in pink and are highly conserved between species and several different GATA family members. Hence, we may have identified a common NLS for the GATA family members.

To gain further insight into how these four amino acids interact with importin β and DNA, we investigated the crystal struc-
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A

|   |   |
|---|---|
| aGATA_6 | CACHTTTTTLWRRNAPEGPVCAAGLYMKHLHHVRFLMKEKQTRKSKPKNI |
| aGATA_5 | CACHTATTTTLWRRNAPEGPVCAAGLYMKHLHHVRFLMKEKQTRKSKPENP |
| aGATA_4 | CACQTTTTLWRRNAPEGPVCAAGLYMKHLHHVRFLMKEKQTRKSKPKNL |
| aGATA_1 | CTCQTTTTLWRRNAEDPVCAAGLTYKHLHVRFLMKEKQTRKSFKAGK |
| rGATA_1 | CTCQTTTTLWRRNAEDPVCAAGLTYKHLHVRFLMKEKQTRKSKPASG |
| hGATA_1 | CTCQTTTTLWRRNAEDPVCAAGLTYKHLHVRFLMKEKQTRKSKPASG |
| cGATA_1 | CTCQTTTTLWRRNAGDPVCAAGLTYKHLHVRFLMKEKQTRKSKPASG |
| dGATA_1 | CACHTTTLWRRNAPEGPVCAAGLTKHLHVRFLMKEKQTRKSKPKNS |

FIGURE 11. Protein sequence alignment of GATA family members and three-dimensional structure of the cGATA-1 DBD. A, Clustal W was used to align the protein sequences of GATA family members. The four crucial residues involved in GATA-4 nuclear import are shown in bold pink and are conserved among family members and species. B, three-dimensional structure of the DBD of cGATA-1 using Deepview/Swiss-Pdb Viewer v3.7. The four basic residues involved in nuclear localization are shown in pink, located either side of the DNA/importin β-binding groove.

The crystal structure of chicken GATA-1 complexed to DNA (Fig. 11B) because the crystal structure of GATA-4 has not yet been solved. As seen by the three-dimensional structure, the four crucial residues are located on either side of the DNA-binding groove (which is also the importin β-binding region). This ensures a stable interaction between GATA-4 and DNA or GATA-4 and importin β. All four residues need to be simultaneously mutated to abolish nuclear import of GATA-4, with individual mutations being insufficient. It is conceivable that nuclear import of GATA-4 proceeds despite these single mutations because of the significance of GATA-4 nuclear transport for gene transcription and regulation. As such, multiple mutations are required to completely abolish nuclear transport, as has been observed for other proteins (33).

We showed that the NLS of GATA-4 is functional in two different cell types, HeLa cells and cardiac myocytes (Figs. 1 and 4A, respectively). However, constructs that did not contain a functional NLS were evenly distributed throughout both the nucleus and cytoplasm of HeLa cells but localized exclusively to the cytoplasm of cardiac myocytes. Generally, larger proteins (>40 kDa), such as the constructs used in our study, are excluded from the nucleus in the absence of an NLS (34). However, it is possible that the conformation of GATA-4 allows it to passively diffuse through the 9-nm diameter of the NPC (35), as has been observed with other large proteins (36). This would explain the presence of GATA-4-GFP in the nucleus of HeLa cells, even in the absence of an intact NLS. The dissimilarity observed in cardiac myocytes could be due to differences in the NPCs of diverse cell types, allowing for variations in the size limit of passively diffusible proteins. However, this is unlikely because NPCs are generally well conserved between eukaryotes (37). An alternative reason for the difference between GATA-4 localization in HeLa cells and cardiac myocytes is the presence of a NES that is functional only in cardiac myocytes and not in HeLa cells. Indeed, treatment with the CRM1 inhibitor, LMB, resulted in an even distribution of those GFP fusion proteins lacking an intact NLS in cardiac myocytes (Fig. 4B). This suggested that the exportin CRM1, required for GATA-4 export, may have a different specificity in HeLa cells, or other components of the nuclear export machinery may be lacking in these cells. Indeed, diverse tissues and cell types express different levels and subtypes of importins and exportins. This has been well documented for importin α (38, 39), but little is known about intracellular levels of exportins and other importin β homologues. In fact, not all importins and exportins are ubiquitously expressed. For example, importin α is absent from the heart, spleen, and kidney (39), whereas importin α1 is abundant in heart, testis, skeletal muscle, and ovary (38). Moreover, protein phosphorylation and dephosphorylation have been shown to regulate import and export (40, 41). This process is mediated by different signaling pathways via many different kinases and phosphatases that may be functional in cardiac myocytes and not in HeLa cells. Interestingly, treatment with LMB had no effect on constructs that contained a functional NLS. This deviates from results observed by Morisco et al. (42) demonstrating an increase in nuclear GATA-4 accumulation. This difference is most likely due to the use of a higher concentration of LMB in the latter study.

The C-terminal zinc finger and adjacent basic domain of GATA-4 has a dual function in both DNA binding and nuclear import. This has been well documented for a wide range of other transcription factors (43, 44), with the NLS of ~90% of DNA-binding proteins coinciding with the DBD (45). This suggests an elegant mechanism to facilitate the execution of nuclear activities. However, because of the overlap of the NLS and DBD, it can be argued that nuclear accumulation of GATA-4 may be attributed to nuclear retention as a result of DNA binding rather than active NLS-mediated transport. However, individual mutants of GATA-4 that failed both to bind DNA in vitro and activate transcription, namely R282A, R283A, R299A, R305A, R311A, and R317A, and R319A (Figs. 8, A and B, and 9), were still actively targeted to the nucleus (Fig. 2B). This implies that the nuclear import function of GATA-4 is distinct from its DNA binding activity, and DNA binding is not required for nuclear import. Moreover, it has been shown previously that zinc fingers can bind importins and confer efficient
nuclear localization of some proteins (46). However, GATA-4 protein with zinc finger destabilizing mutations was still targeted to the nucleus (data not shown), suggesting that an intact zinc finger is not required for GATA-4 nuclear localization.

Nuclear transport receptors continuously shuttle cargo between the nucleus and the cytoplasm (21). It is important that the cargo be released at its destination to allow the importins to be recycled back to the cytoplasm for another round of nuclear import. We have demonstrated two possible mechanisms of cargo release. Firstly, we showed that DNA containing a GATA-binding site and importin β compete for binding to the same GATA-4 region (Fig. 10). This implies that DNA and importin β binding are mutually exclusive for GATA-4, which has also been shown for the yeast transcription factor GAL-4 (47). Hence, DNA binding may play a major role in dissociating has also been shown for the yeast transcription factor GAL-4.

Pryce et al. (22) have also shown that the GATA-4 NLS importin β complex after translocation into the nucleus, as has been suggested for other proteins (43). Secondly, dissociation of the GATA-4 NLS-importin β complex was also observed in the presence of RanGTP (Fig. 5B). Indeed, crystal structure studies of other ncNLS, such as parathyroid hormone-related peptide, complexed to HEAT repeats 1–11 of importin β have shown that this region contained not only the binding site for the ncNLS but also the RanGTP-binding site (49). This overlapping of the ncNLS-binding site with the RanGTP-binding site may be another mechanism for release of cargo, such as GATA-4, inside the nucleus.

In the course of our studies, we observed that mutation of certain residues led to the accumulation of the GATA-4-GFP protein within distinct compartments inside the nucleus (Fig. 2, B and C). For the first time, we documented a correlation between loss of DNA binding activity and this speckled intranuclear distribution. This may represent targeting of transcription factors that are no longer able to bind DNA to specific intranuclear compartments for degradation or some other processing. Accumulation of proteins in subnuclear compartments such as Cajal bodies, splicing factor compartments, the nucleolus, promyelocytic leukemia oncoprotein bodies, RNA polymerase II, and the proteasome have been documented for numerous proteins (50–52), although typically, a specific transport signal directs the wild type protein to these subnuclear compartments. Initial studies suggested that the mutant GATA-4 proteins were not colocalized to proteasomes, splicing factor compartments, or nucleolin (data not shown). Further experiments to elucidate the intranuclear fate of mutant transcription factors, and the mechanisms involved would provide additional novel understanding of gene transcription and regulation.

Nucleocytoplasmic transport and DNA binding activity is paramount for normal cell function, with changes in subcellular localization or loss of DNA binding of transcription factors being implicated in disease and developmental abnormalities (53–55). The current study has generated new insights into the nuclear transport mechanisms of GATA-4 and the residues involved in its DNA binding activity and may provide useful information in the development of novel cardiac-related therapies. Indeed, recently, a peptide that competes with the NLS of calcineurin and hence inhibits calcineurin nuclear import was successfully developed for the prevention of cardiac hypertrophy in vitro (56). Considering that GATA-4 overexpression is sufficient to cause myocardial hypertrophy, understanding the nuclear transport of this transcription factor may potentially lead to novel therapeutic strategies for the prevention of cardiac hypertrophy.

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