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Identification of a Monopartite Sequence in PU.1 Essential for Nuclear Import, DNA-Binding and Transcription of Myeloid-Specific Genes

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Abstract The Ets transcription factor PU.1 is an essential regulator of normal hematopoiesis, especially within the myeloid lineage. As such, endogenous PU.1 predominantly localizes to the nucleus of mammalian cells to facilitate gene regulation. However, to date, little is known regarding the mechanisms of PU.1 nuclear transport. We found, using HeLa and RAW 264.7 macrophage cells, that PU.1 enters the nucleus via passive diffusion and active transport. The latter can be facilitated by: (i) the classical pathway requiring importin α and β; (ii) the non-classical pathway requiring only importin β; or (iii) direct interaction with nucleoporins. A group of six positively charged lysine or arginine residues within the Ets DNA-binding domain was determined to be crucial in active nuclear import. These residues directly interact with importin β to facilitate a predominantly non-classical import pathway. Furthermore, luciferase reporter assays demonstrated that these same six amino acids are crucial for PU.1-mediated transcriptional activation of myeloid-specific genes. Indeed, these residues may represent a consensus sequence vital for nuclear import, DNA-binding and transcriptional activity of Ets family members. By identifying and characterizing the mechanisms of PU.1 nuclear import and the specific amino acids involved, this report may provide insights into the molecular basis of diseases. J. Cell. Biochem. 101: 1456–1474, 2007.

Key words: Ets; hematopoiesis; nuclear localization; PU.1; transcription factor

The transcription factor PU.1 is a pivotal regulator in hematopoiesis, promoting or inhibiting the differentiation of various lineages. It is one of about 35 Ets proteins characterized by the presence of a conserved Ets domain that binds a 10 bp long DNA consensus sequence centered over a 5′-GGAA/T-3′ core [Macleod et al., 1992; Seth et al., 1992; Sementchenko and Watson, 2000]. This Ets region is responsible for regulating gene expression in B lymphocytes, monocytes, and granulocytes by binding to the promoters of genes such as immunoglobulin κ (Ig κ), immunoglobulin λ (Ig λ) and CD11b [Fisher and Scott, 1998; Simon, 1998; Oikawa et al., 1999]. The absence of PU.1 in knockout mice results in failure to develop mature myeloid cells and B-lymphocytes, despite initial commitment to these lineages. This can potentially lead to leukaemogenesis [Scott et al., 1994; Olson et al., 1995; McKercher et al., 1996]. In contrast, overexpression of PU.1 inhibits maturation of the erythroid lineage, leading to erythroleukaemia [Moreau-Gachelin et al., 1988]. Indeed, there has been much suggestion in the literature that PU.1 may be involved in acute myeloid leukemia (AML) [Moreau-Gachelin et al., 1988; Lamandin et al., 2002; Meuller et al., 2002; Vegesna et al., 2002; Dohner et al., 2003; Ley et al., 2003]. Considering the crucial role of PU.1 in cellular development and function, the precise regulation of PU.1 concentration and activity is vital, and nuclear shuttling represents one key mechanism by which this can occur.

The transport of small molecules (<40–60 kDa) into the nucleus is facilitated by passive diffusion, while larger molecules are actively transported [Peters, 1986]. The first step in active nuclear import and export involves the

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presence of a nuclear localization signal (NLS) or nuclear export signal (NES) on the target molecule [Dingwall and Laskey, 1991; Wen et al., 1995]. This signal is recognized by a family of import and export receptors, the importins and exportins [Pollard et al., 1996; Fornerod et al., 1997; Stade et al., 1997]. Typically, NLSs are comprised of a short stretch of basic or positively charged amino acids arranged as either monopartite (a single cluster) or bipartite sequences (two clusters separated by a stretch of approximately 10 amino acids) [Dingwall and Laskey, 1991]. In contrast, NES sequences are typically short stretches of leucine-rich and hydrophobic amino acids [Fukuda et al., 1997].

In the classical nuclear import pathway, the NLS of the target protein is recognized by importin α (IMPα). This is then bound by importin β (IMPβ), which facilitates the transport of the complex through the nuclear pore complex (NPC) [Adam and Adam, 1994; Gorlich et al., 1994; Radu et al., 1995]. The NPC is composed of about 30 distinct proteins called nucleoporins [Rout et al., 2000; Cronshaw et al., 2002]. It is believed that transport of the target molecule bound to the IMPα/β complex is facilitated by an affinity gradient with the different nucleoporins from the cytoplasmic to nuclear side of the NPC. Once inside the nucleus, binding of Ran GTP to IMPβ releases the cargo from the complex [Moore and Blobel, 1993; Floer et al., 1997]. IMPα and β are then recycled for another round of nuclear import.

For some molecules, import is via a non-classical pathway, requiring only IMPβ [Wozniak et al., 1998]. In addition, certain proteins can enter the nucleus independently of any transport carriers, such as β catenin [Fagotto et al., 1998; Yokoya et al., 1999], Erk2 [Matsubayashi et al., 2001; Whitehurst et al., 2002], and unphosphorylated Stat 1 [Marg et al., 2005]. These proteins are thought to interact directly with the nucleoporins for transport.

Since gene transcription occurs in the nucleus, regulating the nuclear import and export of transcription factors is crucial. Indeed, impairment of the nuclear localization of proteins has been associated with a range of disease phenotypes such as Saethre–Chotzen syndrome [Ghouzzi et al., 2000], Holt–Oram syndrome [Fan et al., 2002], tricho-rhino-phalangeal syndrome [Kaiser et al., 2004], Leri–Weill syndrome [Sabherwal et al., 2004], and DiGeorge syndrome [Stoller and Epstein, 2005].

In the current study, we have found that PU.1 can enter the nucleus via passive diffusion and by active transport. The latter can be facilitated by the classical pathway requiring IMPα and β; the non-classical pathway requiring only IMPβ; or via direct interaction with the nucleoporins. We further identified six amino acids within the Ets domain that are essential for recognition of the NLS of PU.1. These residues are also crucial for the DNA-binding activity of PU.1 and hence, its transcriptional activity. Our data may help to elucidate how other Ets proteins traffic to the nucleus to regulate the expression of genes linked to development and disease.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis

To generate GFP-PU.1 fusion protein constructs, the mouse PU.1 sequence (GenBank Accession NM_011355) was obtained by PCR from a pcDNA3 vector, and subcloned into pcDNA-DEST53 and pcDNA-DEST47 vectors (Gateway Technology, Invitrogen, Carlsbad, CA). To generate GST fusion protein constructs, PU.1 was subcloned into pDEST15 (Gateway, Invitrogen). Deletion constructs were generated by PCR-based cloning. Site-directed mutagenesis was performed using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA). The IMPα (PTAC58) and IMPβ (PTAC97) subunit were cloned into the pGEX2T vector. Full length Nup62 in pcDNA3.1/HisB vector and Nup153 in pET28b vector were kindly provided by Professor N. Yaseen (Feinberg School of Medicine, Northwestern University, Chicago, USA).

Cell Culture

Human HeLa cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and the mouse RAW 264.7 macrophage cells were a kind gift from Professor D. Richardson (Department of Pathology, University of Sydney, Australia). Briefly, cells were grown in Dulbecco's minimal essential media (DMEM) or RPMI (Invitrogen, Sydney, Australia), supplemented with 10% fetal bovine serum (Invitrogen). Cells were cultured in an incubator (Thermo Forma,

Marietta, OH) at 37°C in a humidified atmosphere of 5% CO2/95% air.

Immunohistochemistry

HeLa and RAW 264.7 cells were seeded onto cover slips and cultured overnight. Cells were then fixed in 100% methanol and blocked with 5% skim milk in PBS. This was followed by incubation with an anti-PU.1 antibody (sc-352; Santa Cruz, California, CA; 1:50 dilution) and anti-rabbit Alexa Fluor 594 antibody (Invitrogen; 1:2500 dilution). Cover slips were mounted using gelatin glycerol (Sigma) and analyzed using an Olympus confocal microscope (Olympus, Tokyo, Japan).

HeLa cells do not contain endogenous PU.1 (Fig. 1A) and were initially used to examine the intracellular localization of exogenously introduced PU.1 protein. The RAW 264.7 macrophage cells were used as a physiologically relevant cell line that contains endogenous PU.1 located exclusively in the nucleus (Fig. 1B).

Nuclear Localization Studies

HeLa cells were grown on cover slips overnight and transiently transfected with 1–2 μg DNA using Lipofectamine or Lipofectamine 2000 as per manufacturer's instruction (Invitrogen). RAW 264.7 cells were electroporated with 2–5 μg DNA using the Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany) as per manufacturer's protocol, and cultured on coverslips. Cells were analyzed 24–48 h after transfection. Briefly, cells were washed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. Cells were washed again with PBS, mounted using gelatin glycerol (Sigma) and analyzed using an Olympus confocal microscope (Olympus).

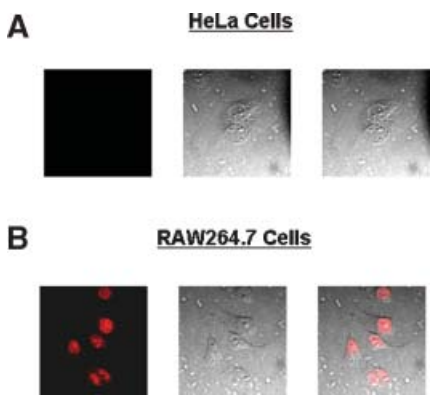

Fig. 1. Localization of endogenous PU.1. (**A**) HeLa cells and (**B**) RAW264.7 cells were stained with an anti-PU.1 antibody and examined using confocal microscopy. Endogenous PU.1 is detected exclusively in the nucleus of RAW264.7 cells.

GST Pull-Down Assays

Constructs in the pDEST53 vector were used to synthesize ^{35}S-labeled proteins using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) in the presence of ^{35}S-methionine. The GST fusion proteins were expressed in *Escherichia coli* strain BL21-A1 (Invitrogen) as described previously [Hu et al., 2005]. GST-pull down assays were performed as detailed previously [Hu et al., 2005]. Briefly, 5 μl of ^{35}S-labeled protein was incubated with 5 μg GST-protein for 1 h at 4°C in 250 μl of pull-down buffer. Following five washes, samples were boiled in the presence of 20% β-mercaptoethanol and loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, gels were dried and autoradiography performed.

Western Blot Analysis

To confirm protein expression in transiently transfected HeLa cells, Western blot analyses were performed as described previously [Kwok and Richardson, 2003]. Briefly, cells were lysed using Passive Cell Lysis Buffer (Promega) or Laemmli buffer containing complete protease inhibitor (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples (<100 μg) containing 20% β-mercaptoethanol were loaded onto a SDS–polyacrylamide gel consisting of 5% stacking and 8% resolving gels. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ) overnight at 4°C.

Membranes were probed using a rabbit anti-PU.1 antibody (sc-352; Santa Cruz, California, CA; 1:200 dilution), followed by an anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz; 1:2000 dilution). Detection was performed using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Wellesley, MA) and exposure to X-ray film.

Luciferase Reporter Assay

The reporter plasmid, pGL3-promoter-4xBSAP-Pip was a kind gift from Professor

Nishizumi (Graduate School of Science, The University of Tokyo, Tokyo, Japan). This reporter contains four copies of the DNA sequence from the BSAP binding motif to the Pip binding motif of the 3′ enhancer of Ig κ light chain gene (κE3′) in the pGL3-promoter vector (Promega) [Hirose et al., 2003]. HeLa cells were transfected with reporter plasmid, control vector phRG-TK (Promega) and expression vector for PU.1 or its mutants using Lipofectamine (Invitrogen). Total amount of DNA was equalized using pcDNA3 backbone. After 48 h, cells were harvested and luciferase activity assessed using the Dual Luciferase Assay Kit (Promega) as per manufacturer's protocol.

Electromobility Shift Assay (EMSA)

The DNA probe contains the PU.1 binding site of the 3′ enhancer of Ig λ light chain gene (λE3′) [Nakano et al., 2005]. The oligonucleotide (5′-GAA AAA GAG AAA TAA AAG GAA GTG AAA CCA AG-3′) and its complement were annealed and end-labeled with [^{32}P]-dATP (Amersham) as described previously [Hu et al., 2005]. PU.1 protein and its derivatives in the pDEST53 vector were synthesized using the TNT T7 Quick Coupled Transcription/ Translation System (Promega) in the absence of ^{35}S-methionine. To confirm protein synthesis, parallel experiments were performed using ^{35}S-methionine. These latter samples were electrophoresed on 8% SDS polyacrylamide gels, dried and autoradiography performed. The protein and DNA probe were allowed to bind and complexes were subjected to electrophoresis on 5% polyacrylamide gels, dried and autoradiography performed. For supershift experiments, samples were incubated with a rabbit anti-PU.1 antibody (1:50 dilution) or anti-GFP antibody (1:50) for 30 min prior to loading onto gels.

Statistical Analyses

Experimental data were compared using Student's *t*-test. Results were considered statistically significant when $P < 0.05$.

RESULTS

Identification of the NLS of PU.1 Within the Ets Domain

PU.1 can be divided into three major domains: the acidic and glutamine rich regions at the N-terminus that play a role in transactivation (aa1–100) [Klemsz and Maki, 1996]; the PEST domain in the middle that is important for protein-protein interaction (aa116–170) [Rogers et al., 1986; Pongubala et al., 1993]; and the Ets domain at the C-terminus that is involved in DNA-binding activity (aa171–257) (Fig. 2A; GenBank Accession 1PUE_E; [Kodandapani et al., 1996; Pio et al., 1996]). To locate the NLS in PU.1, we constructed full length PU.1 (FL) and various deletion fragments tagged with the reporter protein GFP at the N-terminus (Fig. 2A). The deletion constructs were: (i) aa1–170, encompassing the acidic, glutamic, and PEST regions; (ii) aa1–170 fused to aa258–272, representing PU.1 lacking the Ets region (ΔEts); (iii) Ets alone (aa171–257), and (iv) aa168–257 (Fig. 2A). These GFP fusion proteins were expressed by transient transfection of HeLa cells that do not contain endogenous PU.1 (Fig. 1A). Subcellular localization of GFP fusion protein was analyzed by direct immunofluorescence using confocal microscopy.

A construct containing the GFP protein alone (mock) was used as a negative control as GFP lacks a NLS and is small enough to readily diffuse across the nuclear membrane. As expected, GFP was localized in both the nucleus and the cytoplasm (Fig. 2B). Full length PU.1 was only localized to the nucleus of HeLa cells, suggesting active nuclear transport facilitated by the presence of an intact NLS (Fig. 2B). In contrast, the N-terminal fragment of PU.1, aa1–170, localized to both the nucleus and cytoplasm (Fig. 2B). This suggested that active transport was inhibited, but some protein could still enter the nucleus via passive diffusion. Similarly, a construct in which the Ets domain was deleted (ΔEts), failed to accumulate solely in the nucleus (Fig. 2B), suggesting that the Ets region is crucial for active nuclear import, as has been previously documented [Zhong et al., 2005].

To further confirm the above observation, the Ets region alone (aa171–257) was transfected into HeLa cells. However, nuclear accumulation was only observed in ~50% of cells examined (Fig. 2B). Analysis of the amino acid sequence revealed the presence of two positively charged amino acids directly upstream of residue K171, namely, K169 and K170. Hence, the fragment aa168–257 containing these amino acids was examined and nuclear accumulation was clearly observed in 100% of cases (Fig. 2B). This suggested that the residues K169 and K170 are also important determinants of active PU.1

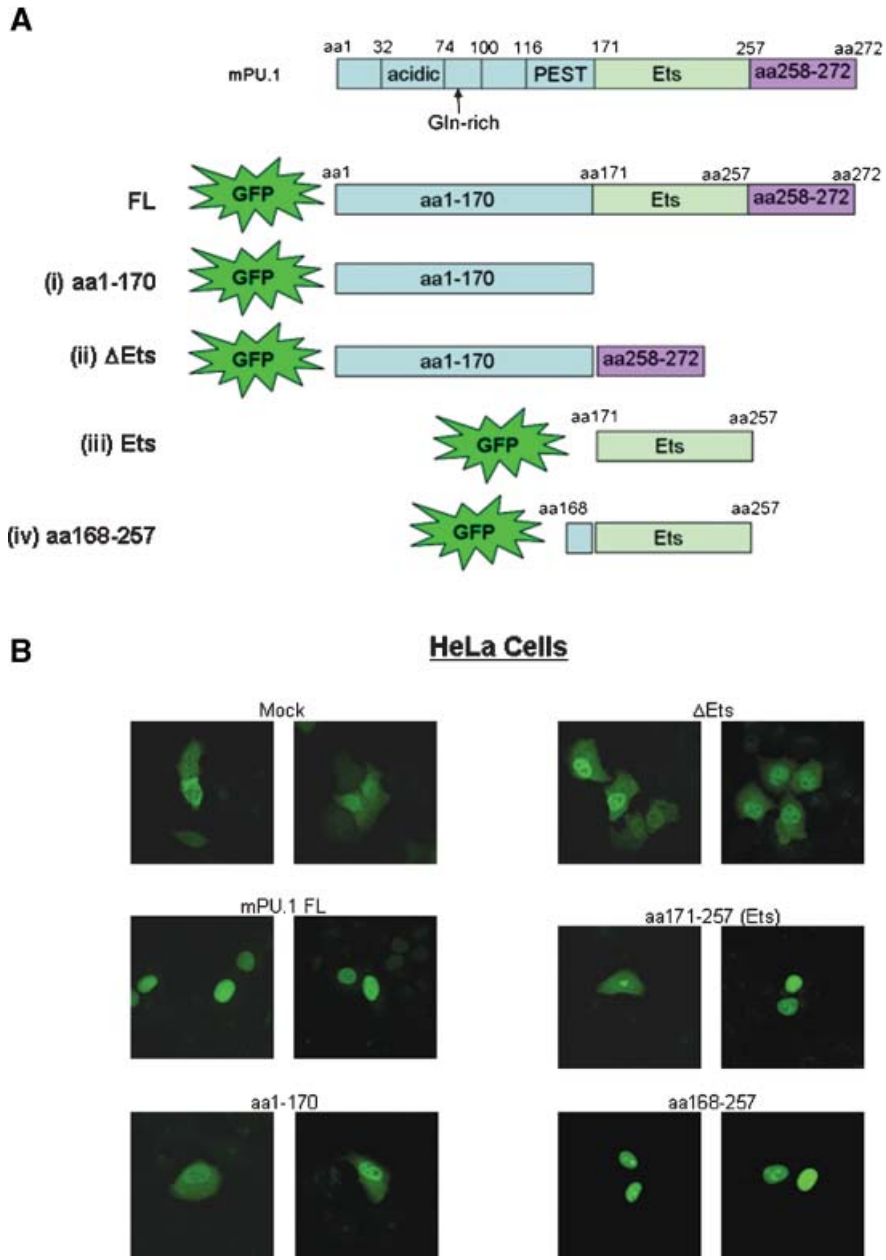

Fig. 2. Identification of the PU.1 NLS using deletion fragments. (**A**) Schematic representation of the PU.1 constructs generated. Full-length PU.1 (FL) and deletion fragments were fused to an N-terminal green fluorescent protein (GFP) tag. The deletion fragments were: (i) aa1–170, encompassing the acidic, glutamic (Gln) and PEST regions; (ii) aa1–170 fused to aa258–272, representing PU.1 lacking the Ets region (ΔEts); (iii) Ets alone (aa171–257); and (iv) aa168–257. (**B**) GFP-tagged full length (FL) and deletion constructs of PU.1 were transfected into HeLa cells using Lipofectamine or Lipofectamine 2000. Nuclear localization of GFP fusion proteins was examined using confocal microscopy after 24–48 h. Only full length (FL) PU.1 and the fragment aa168–257 localize exclusively to the nucleus, indicating the presence of a functional NLS.

transport. Similar results were obtained when GFP was fused at the C-terminal of full length and deletion fragments of PU.1 (data not shown).

From these localization experiments, it can be concluded that the NLS for PU.1 is located within aa168–257 that contains the Ets domain. In the absence of an NLS, GFP-fusion proteins were not exclusively localized in the cytoplasm, but were detected in both the nucleus and cytoplasm. It is likely that this is partially due to passive diffusion of PU.1 and its

constructs into the nucleus, as their predicted molecular weights are less than 60 kDa (~31 kDa for full length PU.1 and ~30 kDa for GFP). However, active transport facilitated by the presence of the NLS results in total nuclear accumulation. None of the fragments examined demonstrated exclusive cytoplasmic GFP accumulation, suggesting that PU.1 does not contain a NES.

Single Mutation of Positively Charged Amino Acids did not Inhibit Nuclear Import of PU.1

To further fine map which amino acids within aa168–257 are crucial for nuclear import of PU.1, single amino acid mutations were introduced. The positively charged lysine (K) or arginine (R) residues were mutated to the neutral alanine (A) in the full-length construct, and transfected into HeLa cells. The eleven PU.1 mutants generated were: K169A, K170A, K219A, R222A, K223A, K224A, K229A, R232A, R235A, K245A, and K249A (Fig. 3).

As shown in Figure 3, mutation of each of the positively charged residues had little effect on the NLS, with the majority of cells displaying nuclear accumulation of the GFP-fusion protein. However, in some cases, GFP could be weakly detected in the cytoplasm (K229A, R232A, R235A). The above results demonstrated that single amino acid mutations within the Ets domain did not abolish nuclear import of PU.1.

Multiple Mutations Are Required to Abolish PU.1 Nuclear Import

Five constructs containing mutations of two positive residues were generated and nuclear localization examined. As shown in Figure 4A, double mutants such as K219A + K229A and R232A + R235A, did not completely inhibit nuclear import, with some cells showing exclusive nuclear accumulation and others showing both cytoplasmic and nuclear localization. Other double mutants (K219A + R232A, K219A + R235A, and K245A + K249A) demonstrated similar GFP localization (data not shown). Disruption of NLS was detected in 7–70% of cells examined for the five different double mutants studied.

Multiple mutants were then generated with various combinations of three, four, five, or six positive residues mutated to alanine (Fig. 4B, C). Upon analysis, it was evident that mutants containing three or more of any of the mutations, K219A, K229A, R232A, R235A, K245A, and K249A, resulted in definitive loss of nuclear GFP accumulation. For example, mutants K219A + R232A + R235A and K229A + R232A + R235A + K245A + K249A resulted in both cytoplasmic and nuclear GFP in more than 90% of cells examined (Fig. 4B and data not shown for other mutants). This indicated that the NLS had been abolished and active nuclear import inhibited. In contrast, mutants containing various combinations of the mutations K169A, K170A, K244A, K247A, and K248A had little effect on the NLS, with clear nuclear GFP accumulation observed for mutants such as K244A + K247A + K248A and K170A + K244A + K247A + K248A (Fig. 4C and data not shown for other mutants).

These results suggested that the six amino acids, K219, K229, R232, R235, K245, and K249, are crucial for nuclear localization of PU.1. At least four of these six residues must be intact to facilitate active nuclear import, as mutation of three or more residues is sufficient to abolish the NLS and inhibit nuclear accumulation. The six residues will be referred to as Group A residues. Other positive residues within the Ets domain, K169, K170, K244, K247, and K248, did not play a significant role in nuclear import. These residues will be referred to as Group B residues.

Nuclear Import of PU.1 in a Macrophage Cell Line, RAW 264.7

The above localization experiments were conducted in HeLa cells due to their higher transfection efficiency and the absence of endogenous PU.1 (Fig. 1A). To confirm these results in a physiologically relevant cell line, we examined the RAW 264.7 macrophage cells that contain endogenous PU.1 localized exclusively in the nucleus (Fig. 1B).

Similar to HeLa cells, transfection of GFP alone (mock) resulted in both nuclear and cytoplasmic localization (Fig. 5A). As expected, full length PU.1 could only be detected in the nucleus (Fig. 5A). A PU.1 construct lacking the Ets domain (ΔEts) resulted in loss of NLS and hence, loss of nuclear accumulation, as observed by both nuclear and cytoplasmic GFP (Fig. 5A). These results confirmed, using macrophages, that the Ets domain is responsible for nuclear import of PU.1.

Again, double mutants of Group A residues such as R232A + R235A and K245A + K249A,

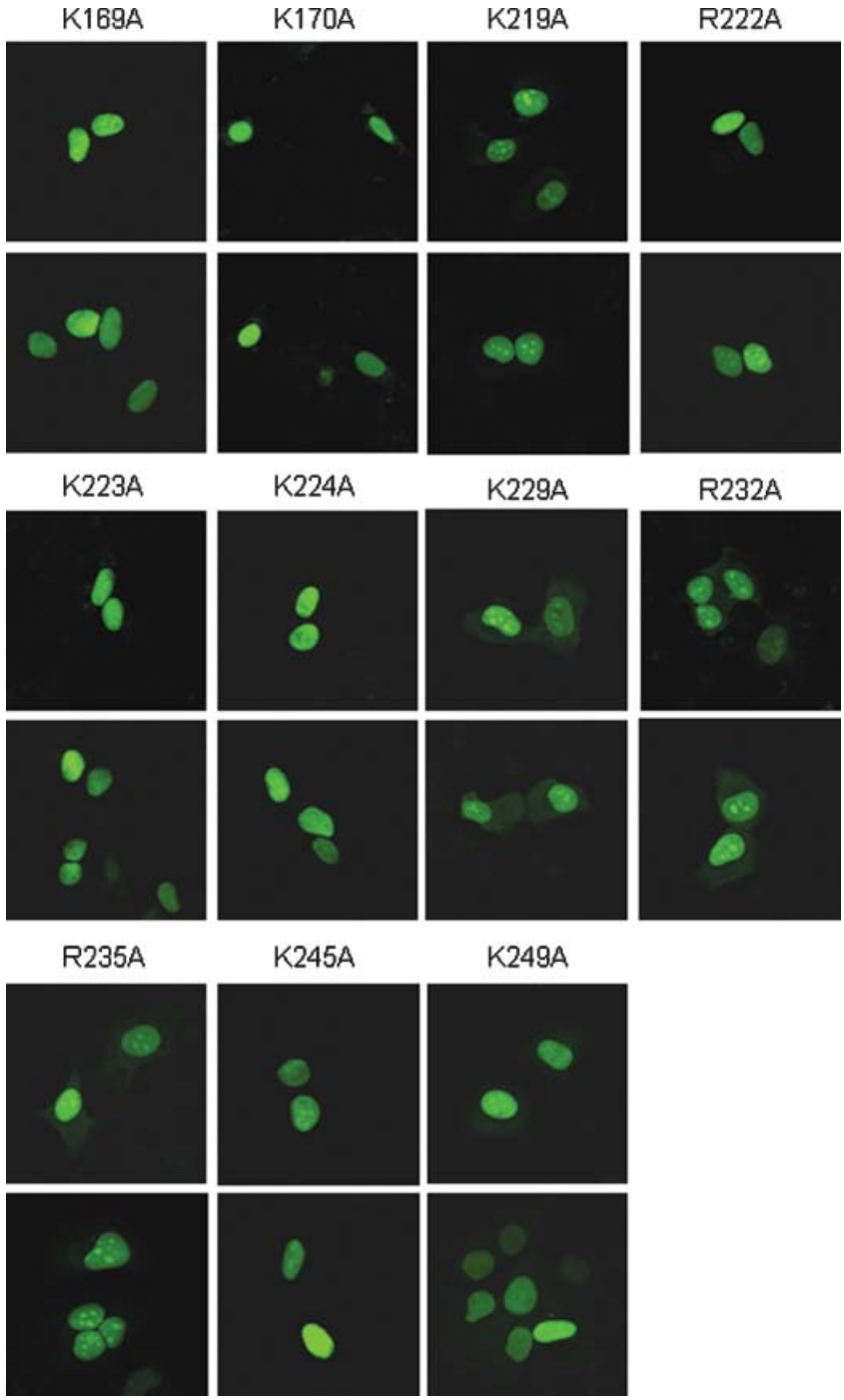

Fig. 3. Mutation of a single amino acid residue in the putative NLS does not affect nuclear import. Mutation of a single lysine (K) or arginine (R) residue within the NLS to alanine (A) was performed by site-directed mutatgenesis. Constructs were then transfected into HeLa cells and nuclear localization examined. GFP nuclear accumulation is observed in all single mutants.

failed to inhibit nuclear import in macrophages (Fig. 5B and data not shown for other mutants). However, multiple mutations of Group A residues inhibited nuclear localization of PU.1 in macrophages, as observed for mutants K219A + K229A + R232A + R235A and R232A + R235A + K245A + K249A (Fig. 5B and data not shown for other mutants). Multiple mutations of Group B

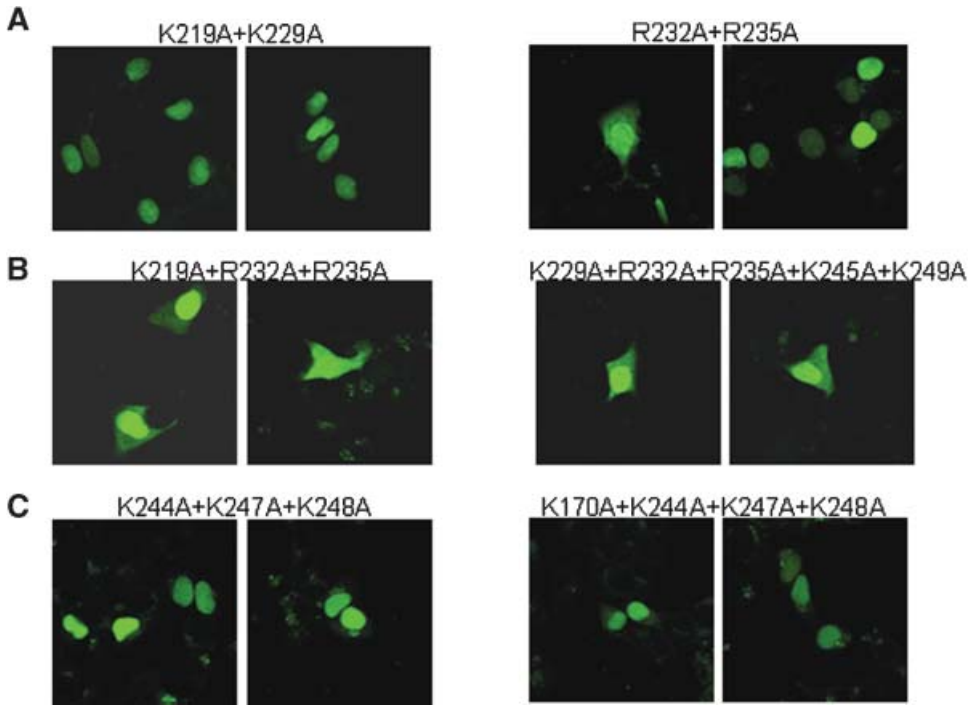

Fig. 4. Mutation of three or more of the Group A residues completely abolishes NLS function and inhibits nuclear import. Constructs containing mutations of two to six positively charged residues were generated by site-directed mutagenesis. Mutant constructs were transfected into HeLa cells and examined using confocal microscopy. (**A**), (**B**) Multiple mutations of Group A residues, namely, K219, K229, R232, R235, K245, K249. Mutation of three or more residues results in loss of nuclear localization. (**C**) Multiple mutations of Group B residues, namely, K169, K170, K244, K247, K248. The NLS cannot be disrupted despite multiple mutation of these amino acids.

residues again failed to disrupt the NLS, with clear nuclear GFP accumulation observed for mutants such as K170A + K244A + K247A + K248A (Fig. 5B and data not shown for other mutants). These results using the macrophage cells as a relevant cell type confirm the observations in HeLa cells.

The Minimal NLS Region

We have identified that the NLS of PU.1 is localized to the Ets domain, and the six Group A residues are crucial components of the NLS. However, the minimal region required to facilitate active nuclear import of PU.1 has not yet been defined.

As shown previously (Fig. 2B), full length PU.1 and the Ets domain spanning aa168–257 were targeted to the nucleus (Fig. 6). The fragment aa168–249 containing all six Group A residues demonstrated nuclear accumulation as expected (Fig. 6). The fragment aa168–235, containing only four of the six residues, was also actively targeted to the nucleus. However, aa168–232 and aa168–219 with only three and one of the Group A residues, respectively, failed to target to the nucleus (Fig. 6). Therefore, the minimal region required for active PU.1 nuclear import is aa168–235, containing four of the six Group A residues. These results confirm the above mutational studies (Fig. 5) demonstrating that at least four of the six amino acids must be intact to actively transport PU.1 to the nucleus.

Mechanism of PU.1 Nuclear Import

Typically, nuclear import is facilitated by the importins, via the classical or non-classical pathway. In the first, the NLS is recognized and bound by IMPα. This is followed by binding of IMPβ and the transit of the complex through the NPC. However, in the non-classical pathway, IMPβ alone binds and transports the cargo into the nucleus. To elucidate the mechanism of PU.1 nuclear import, GST pull-down assays were performed.

IMPα and IMPβ were synthesized as GST-fusion proteins and allowed to interact with ^{35}S-labeled PU.1 protein. As shown in Figure 7A, GST alone failed to bind ^{35}S-PU.1 (lane 1). However, both IMPα and IMPβ bound PU.1,

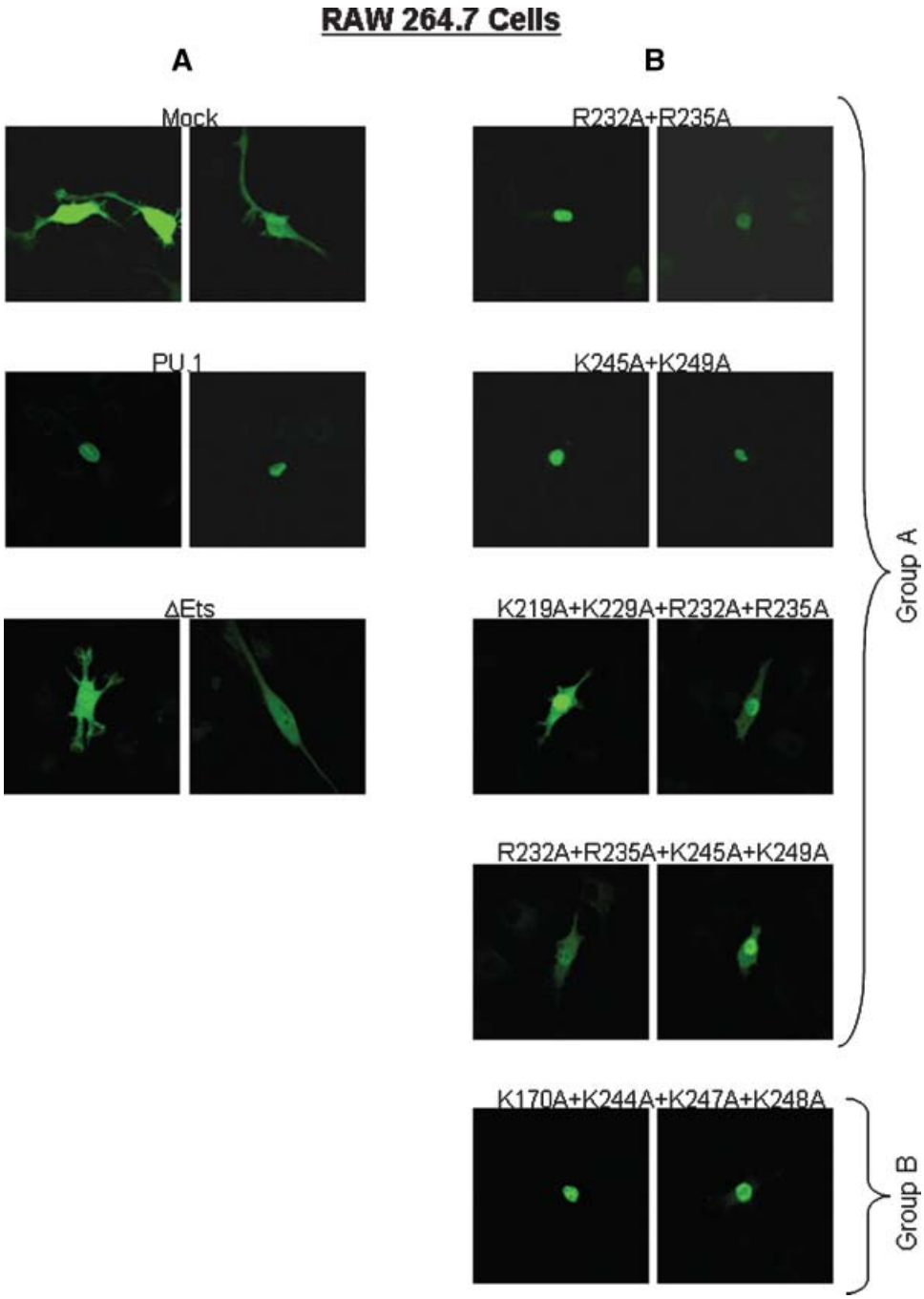

Fig. 5. Nuclear localization of PU.1, its fragments and mutants in RAW 264.7 macrophage cells. The GFP-tagged constructs containing PU.1, its fragments (**A**) and mutants (**B**) were transfected into RAW 264.7 macrophage cells using Nucleofection as described in *Experimental Procedures*. Nuclear localization was determined by confocal microscopy. The deletion fragment ΔEts, and mutants containing more than three Group A residue mutations demonstrate loss of nuclear GFP accumulation.

with stronger binding observed with the latter (Fig. 7A, lanes 2 and 3). This suggests that PU.1 nuclear import is facilitated by both the classical and non-classical pathway, but the latter may predominate. Lane 4 shows 20% input of the ^{35}S-PU.1 protein alone. The absence of the Ets domain (ΔEts) failed to bind IMPβ (Fig. 7B, lane 2) while the Ets domain alone bound to IMPβ (lane 4). This again supports the role of the Ets domain in nuclear import of PU.1

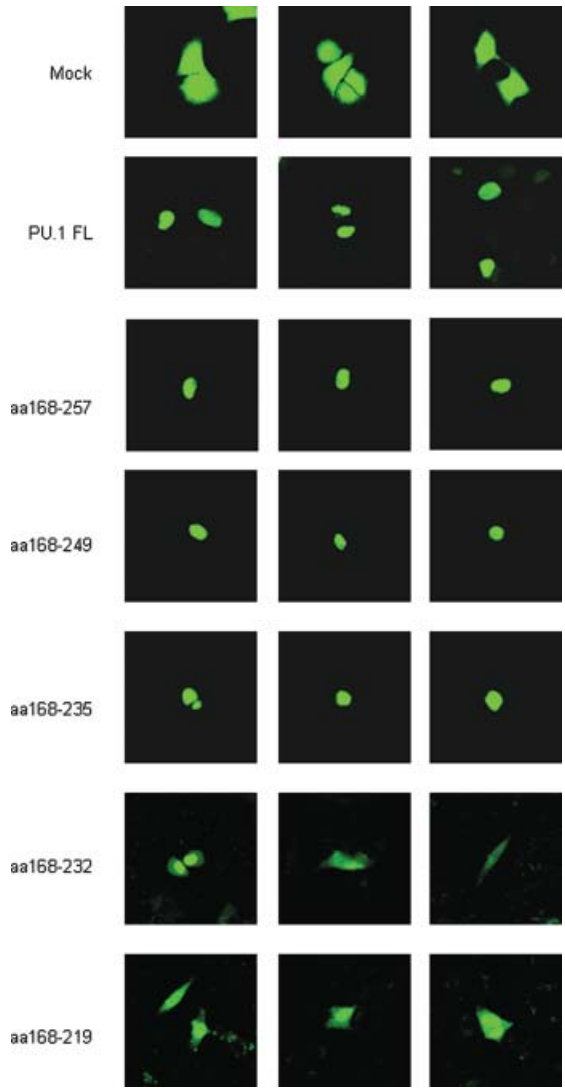

Fig. 6. The minimal NLS region is aa168–235. The GFP-tagged constructs containing PU.1 and its deletion fragments were transfected into HeLa cells and cellular localization examined via confocal microscopy. The minimal region that can be actively targeted to the nucleus is aa168–235.

via binding to importins. Neither fragments bound GST alone (lanes 1 and 3). The 10% input of the ^{35}S-ΔEts and ^{35}S-Ets proteins are shown in lanes 5 and 6. Additional bands observed for PU.1 are likely due to degradation products generated in the TNT system or an internal start site.

To further confirm the above observations, the interaction of PU.1 mutants with IMPβ was examined. In correlation with the nuclear localization results, mutants that do not inhibit nuclear import, such as K170A + K244A + K247A + K248A and K219A + R235A, showed strong binding to IMPβ (Fig. 7C, cf. lane 2 with lanes 4 and 10). In contrast, mutants that inhibit nuclear import, such as R232A + R235A + K245A + K249A and K219A + K229A + R232A + R235A + K245A + K249A, demonstrated much weaker binding to IMPβ (Fig. 7C, cf. lane 2 with lanes 6 and 8). GST alone failed to interact with PU.1 or any of its mutants (lanes 1, 3, 5, 7, and 9). The generation of ^{35}S-PU.1 mutants via the TNT system is demonstrated in Figure 7D, showing 20% of the input used in Figure 7C. These results support the IMPβ-mediated nuclear import of PU.1, and that mutations which abolish nuclear localization are due loss of IMPβ interaction.

To ensure that the lack of interaction between PU.1 mutants and IMPβ was not a non-specific effect due to a loss of three-dimensional protein structure, we examined the ability of these mutants to interact with a known partner of PU.1, namely, GATA-1 [Rekhtman et al., 1999; Zhang et al., 1999; Nerlov et al., 2000; Liew et al., 2006]. Figure 8A shows that ^{35}S-labeled full length PU.1 and the Ets domain bound GST-GATA-1 strongly (lanes 2 and 6). However, a fragment lacking the Ets domain (ΔEts) failed to bind GATA-1 (Fig. 8A, lane 4). This is expected as the Ets domain has been documented to be the region that physically interacts with GATA-1 [Rekhtman et al., 1999; Zhang et al., 1999; Nerlov et al., 2000; Liew et al., 2006]. None of the ^{35}S-PU.1 fragments bound GST alone (Fig. 8A, lanes 1, 3 and 5). Figure 8B shows 10% input used in Figure 8A.

Both mutants that fail to bind IMPβ and fail to enter the nucleus, such as K229A + R232A + R235A + K245A + K249A and K229A + R232A + R235A, as well as mutants that can bind IMPβ and actively enter the nucleus, such as K224A + R232A + R235A, were found to bind GATA-1 (Fig. 8C, lanes 3–5). This suggested that the mutations have not altered three-dimensional structure sufficiently to render loss of all protein–protein interaction, but rather, the loss of IMPβ binding is a specific effect. The GATA-1 binding activity of mutant K229A + R232A + R235A + K245A + K249A was weaker compared to wildtype (Fig. 8C, cf. lanes 2 and 3). This is not surprising as the Ets domain is responsible for interacting with GATA-1 and mutation of five positively charged residues may alter the efficiency of GATA-1-binding activity. However, alterations in three-dimensional

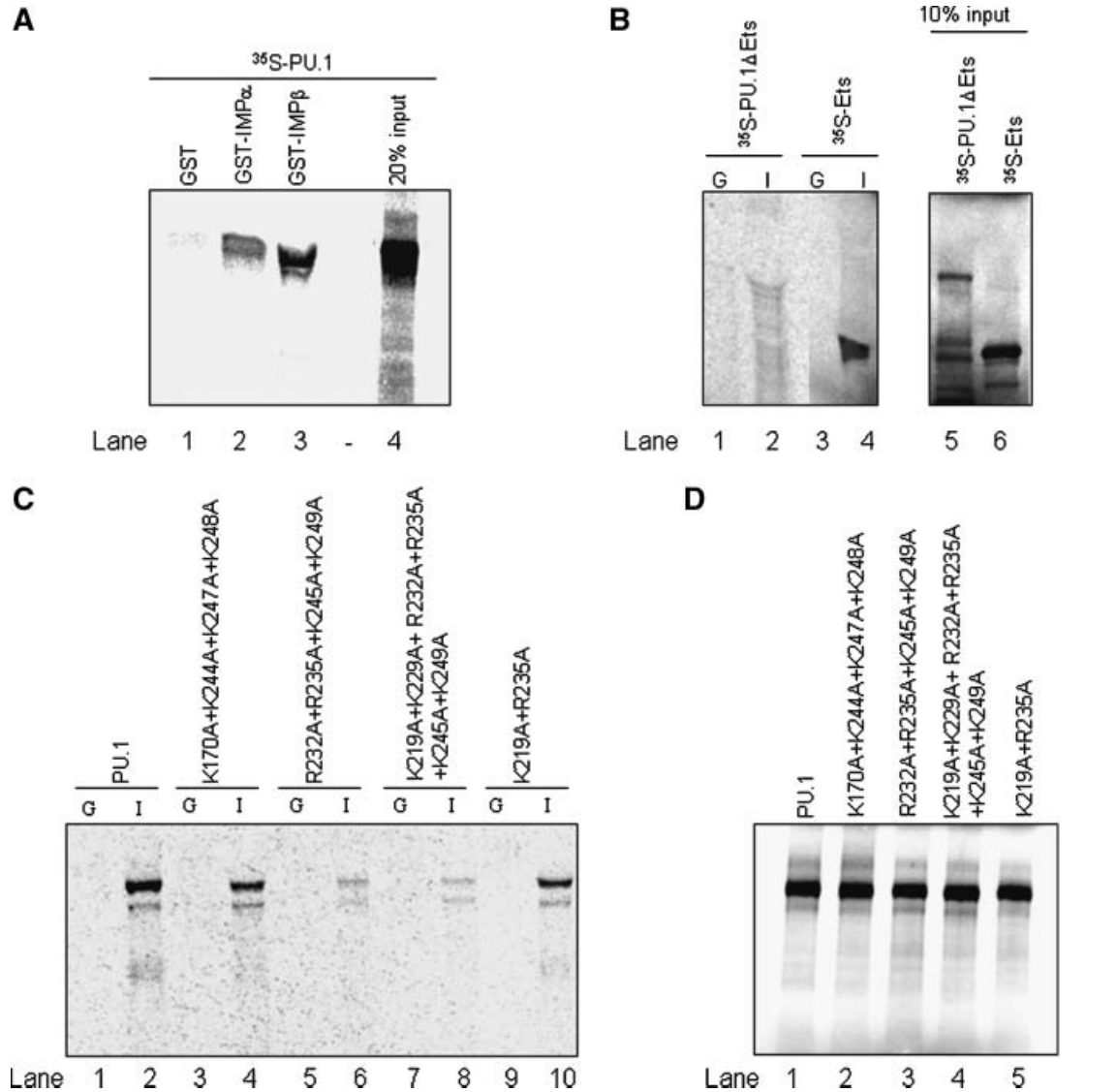

Fig. 7. PU.1 interacts with IMPα and IMPβ via the six Group A residues. (**A**) GST-IMPα and β were generated and allowed to interact with TNT-synthesized ^{35}S-PU.1. GST pull-down assays and autoradiography were performed. (**B**) ^{35}S-PU.1 ΔEts and ^{35}S-PU.1 Ets were generated using the TNT system. Interaction with GST alone (G) or with GST-IMPβ (I) was determined using the pull-down assay and autoradiography. 10% input of ^{35}S-PU.1 fragments is shown. (**C**) Pull-down assay of ^{35}S-PU.1 protein and its derivatives interacting with GST alone (G) or with GST-IMPβ (I). (**D**) 20% input of ^{35}S-PU.1 and its derivatives used in (C). Typical experiment of two performed.

structure were not sufficient to completely abolish interaction with GATA-1. Lane 6 shows 10% input of ^{35}S-labeled GATA-1. Figure 8D shows protein expression of GST and GST-fusion PU.1 mutants.

Recently, it was shown that PU.1 interacts with nucleoporins for import, specifically, Nup62 and Nup153 [Zhong et al., 2005]. By GST-pull down assays we were able to confirm that PU.1 does indeed bind Nup62 and Nup153 (data not shown). However, results with PU.1 mutants were inconclusive, there being no definitive correlation between mutants that fail to accumulate in the nucleus and binding to Nup62 or Nup153 (data not shown). This suggested that nucleoporin-dependent import is not facilitated by the Group A or Group B residues. However, as there are more than 30 different nucleoporins making up the NPC, it cannot be ruled out that other nucleoporins, besides Nup62 and Nup153, may be crucial regulators for PU.1 entry. Similarly, it cannot

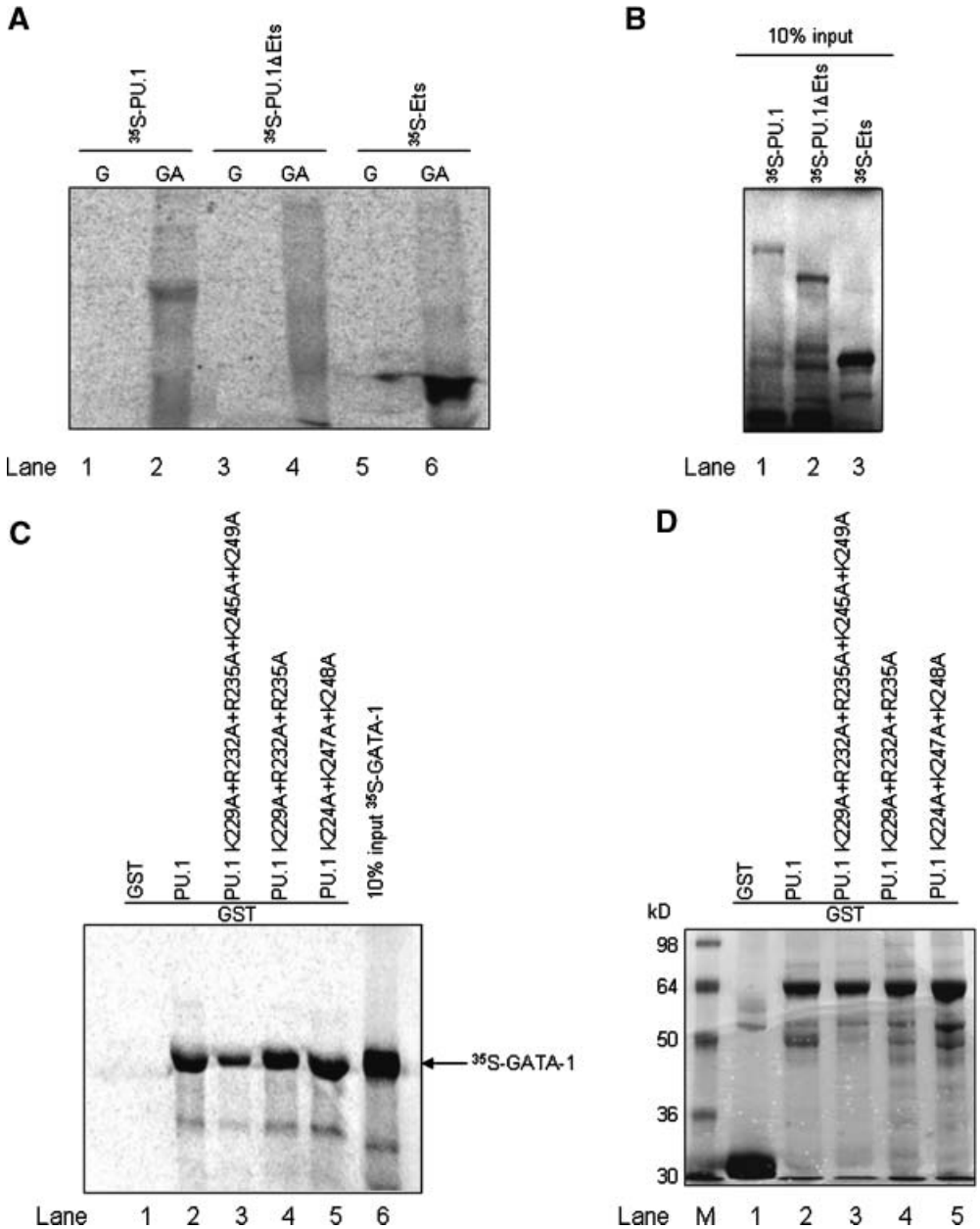

Fig. 8. Mutations in PU.1 do not affect its ability to interaction with GATA-1. (**A**) ^{35}S-PU.1 and its fragments were generated and allowed to interact with GST alone (G) or with GST-GATA-1 (GA). GST pull-down assays and autoradiography were performed. (**B**) 10% input of ^{35}S-PU.1 and its fragments used in (A). (**C**) ^{35}S-GATA-1 was generated using the TNT system. Interaction with GST alone, GST-PU.1 and its mutants was determined using the pull-down assay and autoradiography. (**D**) Protein gel stained with SeeBlue to demonstrate the synthesis of GST fusion proteins. M represents protein marker. Typical experiment of two performed.

be excluded that other amino acid residues, besides the ones examined in this study, may interact directly with nucleoporins to facilitate import.

From the above results, it is evident that active nuclear transport of PU.1 can be facilitated by three mechanisms: classical import via IMPα and β; non-classical import via IMPβ only; and importin-independent import by direct interaction with nucleoporins. However, the non-classical pathway is the dominant mechanism, and requires the interaction of Group A residues, namely, K219, K229, R232, R235, K245, and K249, with IMPβ.

Transcriptional Activation by PU.1

To determine whether mutation of the positive residues affects functional activity of PU.1, the ability of PU.1 to activate transcription of lymphocytic genes was examined using Luciferase Assays. The promoter region of the 3′ enhancer of Ig κ light chain gene was cloned upstream of the luciferase reporter gene. Cells were transiently transfected with the reporter gene, together with full length PU.1, its fragments or mutants and luciferase activity assessed. Transfection efficiency was normalized with the control vector, phRG-TK (Promega). Western blot analyses on transfected HeLa cells demonstrated that all the PU.1 constructs were translated into proteins (Fig. 9 insets).

As expected, the absence of the Ets DNA-binding domain in fragments aa1–170 and ΔEts resulted in markedly reduced transcriptional activity when compared to wild type PU.1 (Fig. 9A). However, the Ets region, as shown in Ets alone and aa168–257, also significantly reduced transcription activation (Fig. 9A). This result is expected due to the absence of the acidic and glutamine-rich regions that are responsible for transactivation, and hence confirms that the transcriptional activity of PU.1 depends on the concerted presence of all domains.

Single mutations of Group A residues markedly reduced transcriptional activation by PU.1 (Fig. 9A). However, single mutation of Group B residues, such as K169A and R224A, only slightly reduced transactivation (Fig. 9A). As expected, multiple mutations of Group A residues significantly decreased luciferase activity (Fig. 9B). Interestingly, multiple mutations of Group B residues also significantly decreased transactivation (Fig. 9B).

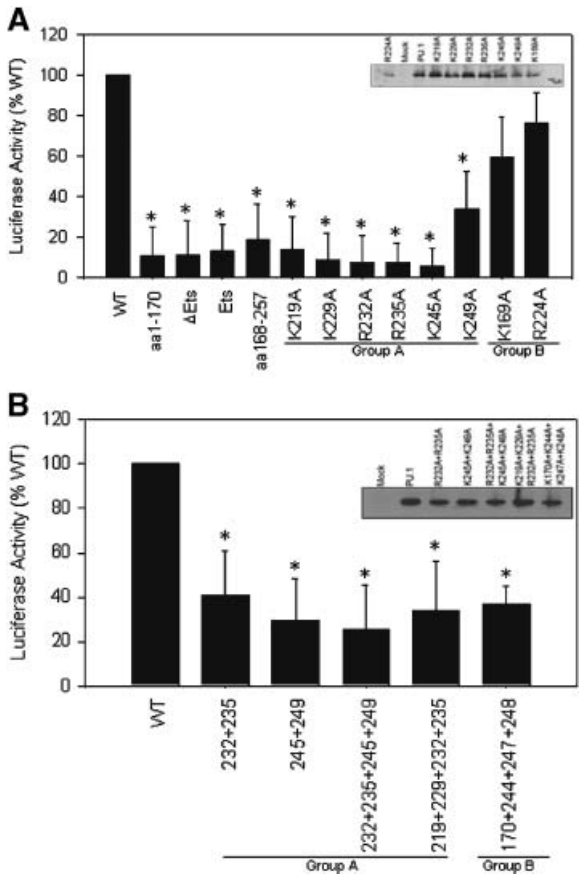

Fig. 9. The six Group A residues are crucial for transcriptional activity of PU.1. Cells were transiently transfected with the reporter plasmid, control reporter plasmid and expression vectors for PU.1 and its mutants. The reporter plasmid contains four copies of the PU.1 binding motif of the 3′ enhancer of Ig κ light chain gene (κE3′) upstream of the firefly luciferase gene in the pGL3-promoter vector. To compensate for transfection efficiency, results were normalized by determination of the *Renilla* activity of the control vector, phRG-TK. Luciferase activity detected using a mock vector was considered background reading. Activity was determined using the Dual Luciferase Assay Kit (Promega) 48 h after transfection. Data are expressed as a percentage of wild-type (WT) PU.1 activity. Insets show Western blot analyses performed to ensure all mutant PU.1 proteins were expressed in transfected cells. (**A**) Transcriptional activity of PU.1, its fragments and single mutants. (**B**) Transcriptional activity of wild type PU.1 and PU.1 containing multiple mutations. Results are a mean ± s.d. of six or seven separate experiments performed. (*$P < 0.002$).

DNA-binding Activity of PU.1 Fragments and Mutants

While four of the Group A residues (K219, R232, R235, and K245) are known to be important for binding DNA [Kodandapani et al., 1996], we further investigated the DNA-binding activity of the other Group A and B residues as they are located in the Ets domain. Electromobility shift assays (EMSAs) were performed with the PU.1 binding site of the 3′ enhancer of Ig λ light chain gene (λE3′) as DNA probe. PU.1 protein and its fragments were synthesized using the TNT system in rabbit reticulocyte lysates and visualized to ensure protein synthesis (insets of Fig. 10). As can be seen in Figure 10A,B insets, two protein bands can be detected for most PU.1 samples. This observation of multiple bands has previously been documented for PU.1 and is likely due to degradation products, an internal start site, or phosphorylation [Lloberas et al., 1999]. To

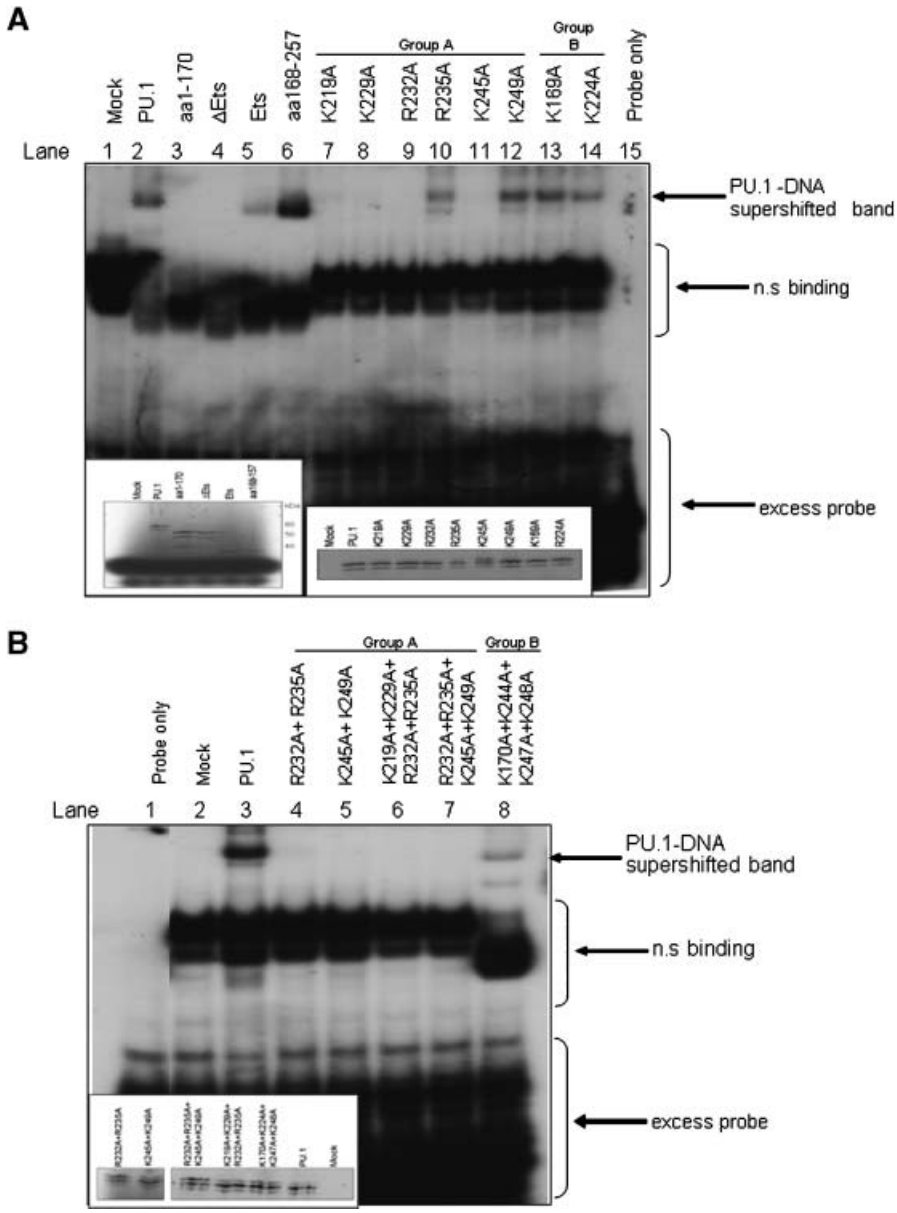

Fig. 10. DNA-binding activity of PU.1, its fragments and mutants. Wild-type PU.1 and its derivatives in the pDEST53 vector were generated using the TNT system (insets). EMSA was performed using a ^{32}P-labeled probe that contains the PU.1 binding site of the 3′ enhancer of Ig λ light chain gene (λE3′). Supershift was performed using an anti-GFP or anti-PU.1 antibody. (**A**) DNA-binding activity of full length PU.1, deletion fragments and single mutants. (**B**) DNA-binding activity of PU.1 containing multiple mutations. Typical experiment of four performed.

visualize protein-DNA binding clearly in the EMSA gels, all samples were supershifted with an anti-PU.1 or anti-GFP antibody.

As expected, fragments lacking the Ets domain, that is, aa1–170 and ΔEts, failed to bind DNA compared to wild-type PU.1 (Fig. 10A, cf. lane 2 with lanes 3 and 4). The Ets domain only bound DNA weakly (lane 5). However, aa168–257 that includes the two positive amino acids K169 and K170, demonstrated strong DNA-binding activity compared to Ets alone (cf. lanes 5 and 6). This is interesting, as these residues were also found to be important in conferring absolute nuclear accumulation (Fig. 2B). Moreover, it was consistently observed that DNA-binding of aa168–257 was stronger than that of wild type PU.1 (cf. lanes 2 and 6) despite equal protein loading. It is

possible that some inhibitory or regulatory element may be located outside of aa168–257, or the full-length protein may provide some steric hindrance to DNA-binding, as documented for other DNA-binding proteins [Westman et al., 2003; Bao et al., 2004].

Although single mutations of Group A residues did not inhibit nuclear import of PU.1, four of the six amino acids were crucial for DNA-binding activity. The mutants K219A, K229A, R232A, and K245A completely abolished DNA-binding (Fig. 10A, lanes 7–9, and 11). Mutation of residues R235A and K249A reduced or had little effect on DNA-binding, respectively (lanes 10 and 12). Single mutations of Group B residues, such as K169A and K224A, failed to abolish DNA-binding (lanes 13 and 14). The above results argue against nuclear retention as a possible explanation of nuclear localization, as mutants that fail to bind DNA can still be fully targeted to the nucleus (Fig. 3). This supports nuclear accumulation of PU.1 mediated by an active and functional NLS.

Mutation of two or more of the Group A residues completely abolished DNA-binding activity compared to wild-type (Fig. 10B, cf. lane 3 with lanes 4–7). Multiple mutations of Group B residues, such as K170A + K244A + K247A + K248A, also significantly reduced DNA-binding activity, although very weak binding could still be observed (cf. lanes 3 and 8). This is in agreement with the reduced transcriptional activity observed (Fig. 9B).

From the above results, it is obvious that the DNA-binding activity of PU.1 is stringently controlled. A single mutation of the Group A residues is sufficient to inhibit DNA-binding (except for K249), while double mutations totally abolish DNA-binding activity. This correlates with the significantly reduced transcriptional activity observed with single and multiple mutations in Group A residues (Fig. 9). Indeed, multiple mutations of Group B residues also decreased DNA-binding activity. Again, this agrees with the reduced transcriptional activity observed (Fig. 9). Our results suggest that the six Group A residues are vital for PU.1 function as they regulate nuclear import, DNA-binding as well as transcriptional activity.

DISCUSSION

The Ets transcription factor PU.1 is crucial in regulating the expression of genes involved in the development and normal cell function of the hematopoietic system. Absence of PU.1 can lead to leukaemogenesis, while overexpression of PU.1 can induce erythroleukaemia [Moreau-Gachelin et al., 1988; Scott et al., 1994; Olson et al., 1995; McKercher et al., 1996]. Indeed, PU.1 has been linked to AML [Moreau-Gachelin et al., 1988]. As such, the transport of PU.1 into the nucleus where gene transcription occurs must be precisely controlled. Despite this, the mechanism of PU.1 nuclear import is not fully understood.

The current study is the first to investigate the detailed mechanisms of PU.1 nuclear transport in both HeLa cells and macrophages as a physiologically relevant cell type. During the course of this present investigation, a study using HeLa cells documented the presence of an NLS in the Ets domain and a nucleoporin-mediated pathway of PU.1 nuclear import [Zhong et al., 2005]. In our work, we demonstrate that PU.1 can enter the nucleus via passive diffusion as well as active transport. In agreement with Zhong et al. [2005], we confirmed that PU.1 directly interacts with nucleoporins. However, studies examining the interaction between PU.1 mutants and nucleoporins were inconclusive. Furthermore, we showed that import is also mediated by interaction with both IMPα and IMPβ, with a preference for IMPβ, thus representing the classical and non-classical import pathways, respectively. We fine mapped the six crucial amino acids within the Ets region required for IMPβ-mediated nuclear import. The vital role of these residues in PU.1 function is further exemplified by their importance in DNA-binding and transcriptional gene regulation.

The colocalization of the NLS and the DNA-binding region in PU.1 has been documented for numerous other transcription factors, and may suggest evolutionary clustering of these domains to facilitate the execution of nuclear activities [Sabherwal et al., 2004; Hu et al., 2005; Stoller and Epstein, 2005]. The two positive residues upstream of the Ets domain in PU.1 (K169 and K170) were found to be important for conferring full function to this region, as demonstrated in our localization studies and EMSAs. Indeed, some authors have included these residues as part of the Ets region [Moreau-Gachelin, 1994]. The basic nature of these amino acids may support the minimal Ets region, for example, by promoting the exposure

of the NLS to the protein surface to facilitate binding of the NLS receptor.

We identified six positively charged amino acids within the Ets region, K219, K229, R232, R235, K245, and K249, which are crucial for nuclear import of PU.1. These are termed the Group A residues. Other positive amino acids in the Ets domain were also studied, namely, K169, K170, K244, K247, and K248. However, these do not significantly affect nuclear accumulation and were termed Group B residues. Indeed, the Group A residues are responsible for direct interaction with IMPβ to facilitate a predominantly non-classical import pathway. As seen by the three dimensional structure (Fig. 11A), these six residues all physically lie in the same plane, and thus can accommodate the binding of proteins such as IMPβ over an extended interface. At least four of these six residues must be intact to facilitate nuclear import as mutation of three or more of the six Group A amino acids is sufficient to abolish the NLS of PU.1 and thus, inhibit nuclear accumulation. Since nuclear import of transcription factors is essential to enable gene transcription, it is plausible that there exist mechanisms allowing nuclear import to continue despite single mutations in the NLS. Hence, it is possible that the compensatory mechanism is such that multiple missense mutations must occur to fully inhibit nuclear transport of PU.1, as has been observed for other proteins [Hu et al., 2005; Liu et al., 2006].

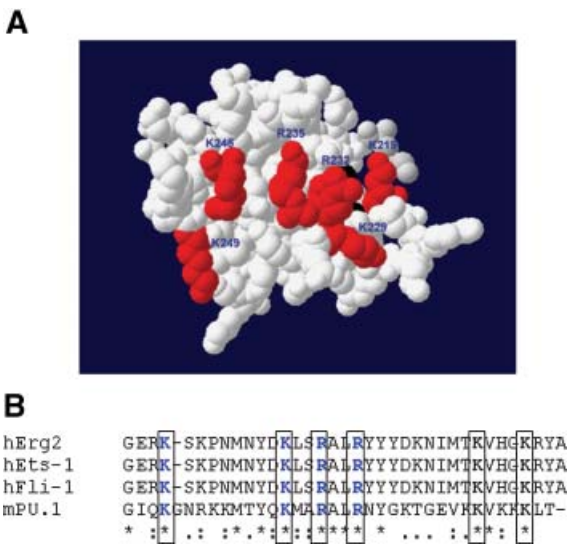

Fig. 11. Three-dimensional structure of PU.1 Ets domain and protein sequence alignment of Ets transcription factors. (**A**) Three-dimensional structure of the Ets domain of PU.1 using Deepview/Swiss-Pdb Viewer v3.7. The six Group A residues shown in red lie in the same plane. (**B**) The protein sequences for Ets family members were aligned using Clustal W. The six crucial residues involved in PU.1 nuclear import are shown in bold. The corresponding conserved residues in Fli-1, Erg2, and Ets-1 are also shown in bold and boxed.

The same six Group A amino acids are also crucial for DNA-binding activity of PU.1. In agreement with previous investigations [Kodandapani et al., 1996], single mutations of Group A residues resulted in complete inhibition or significantly reduced DNA-binding with the exception of mutant K249A, which showed the strongest binding of all the mutants. This is not surprising as crystal structure studies have shown that R232 and R235 interact directly with DNA, while residue K245 binds upstream of the core GGAA sequence, and K219 binds downstream of GGAA on the opposite DNA strand. However, K249 does not directly interact with DNA [Kodandapani et al., 1996]. It was surprising that mutation of R235 still bound DNA weakly according to our EMSA results (Fig. 10A) despite evidence from crystal structure studies demonstrating that R235 directly interacts with DNA [Kodandapani et al., 1996]. It is important to note that in our studies, residues were mutated to alanine which may not have been sufficient to completely abolish the PU.1–DNA interaction at residue R235. Furthermore, in contrast to published data [Kodandapani et al., 1996], we also found that K229 is important for DNA-binding. The above variations in results could be due to a difference in the DNA target used or a difference between crystal structure analyses and EMSAs. The Group A mutants also markedly decreased transcriptional activity of PU.1, further confirming that these residues are indispensable for PU.1 function.

It is of interest to note that a possible explanation for nuclear accumulation of a protein may be that of nuclear retention as a result of DNA-binding. However, this is not likely for PU.1, as mutants that failed to bind DNA could still be localized to the nucleus (e.g., K219A, K229A, R232A + R235A (see Figs. 3, 4, and 10)). This indicates that nuclear accumulation occurred as a result of active NLS-mediated import.

The control of nuclear transport is a crucial regulator of differentiation and proliferation. Indeed, changes in subcellular localization of certain molecules, including transcription

factors, have been associated with disease phenotypes such as cancers and developmental malformations [Ghouzzi et al., 2000; Fan et al., 2002; Kaiser et al., 2004; Sabherwal et al., 2004; Stoller and Epstein, 2005]. In addition, there has been much suggestion that PU.1 may be a suppressor of myeloid leukemogenesis. However, attempts to detect mutations in the PU.1 gene in patients with AML have led to conflicting results [Lamandin et al., 2002; Meuller et al., 2002; Vegesna et al., 2002; Dohner et al., 2003; Ley et al., 2003]. While larger studies must be conducted to confirm the role of mutant PU.1 in AML, it is possible that the mutations we identified that cause altered nuclear localization could be associated with AML. In fact, a study examining radiation-induced AML in mice detected 34 mutant PU.1 alleles from 39 samples. All mutations were located in the Ets domain and 31 of these involved a missense mutation of residue R235 [Cook et al., 2004], one of the Group A amino acids we identified to be involved in PU.1 nuclear import. Furthermore, many alleles showed multiple missense mutations [Cook et al., 2004], giving support to our observation that multiple mutations are required to inhibit nuclear import. However, while single mutations do not fully abolish nuclear import, it cannot be ruled out that this may lead to significant downstream effects, such as reduced transcriptional activation of myeloid-specific genes (Fig. 9). Hence, this study provides important data for further studies examining a possible role of PU.1 mutations in AML, and altered nuclear import as a possible mechanism of disease.

In our previous study, we investigated the NLS of another Ets family member, Fli-1, which is important in megakaryopoiesis. Two NLSs were identified—NLS1 at the N-terminal domain and NLS2 in the Ets region [Hu et al., 2005]. Comparing the NLS region of PU.1 with that of Fli-1, we found high homology, with all six Group A residues involved in PU.1 nuclear import conserved in Fli-1 NLS2 (Fig. 11B). These same six residues were also critical for NLS2-mediated nuclear import of Fli-1, also via preferential interaction with IMPβ [Hu et al., 2005]. In fact, the six residues are found in other Ets transcription factors such as Erg2 and Ets-1 (Fig. 11B). These residues may represent a consensus sequence vital for nuclear import, DNA-binding and transcriptional activity of Ets family members.

As numerous diseases have been attributed to the mis-localization of transcription factors including SHOX, Tbx1, Tbx5 as well as AML1 [Reamon-Buettner and Borlak, 2004; Sabherwal et al., 2004; Stoller and Epstein, 2005; Vradii et al., 2005], it is possible that missense mutations in the NLS of Ets transcription factors may have structural or functional consequences resulting in disease phenotypes. This current study forms a firm basis for further investigations examining the nuclear import of Ets transcription factors. Understanding the mechanisms of nuclear targeting may provide crucial information on the molecular basis of certain diseases.

The current report provides the first detailed analysis of the nuclear import mechanisms of PU.1. Specifically, the six residues, K219, K229, R232, R235, K245, and K249, are crucial in the NLS and interact with IMPβ to facilitate import predominantly via the non-classical pathway. These same residues are vital for PU.1-mediated transcriptional activation of myeloid-specific genes. This information may help to elucidate how other Ets proteins traffic to the nucleus to regulate expression of genes involved in development and tumor progression.

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