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Identification of a functional nuclear localization signal within the human USP22 protein

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ABSTRACT

Ubiquitin-specific processing enzyme 22 (USP22), a member of the deubiquitinase family, is over-expressed in most human cancers and has been implicated in tumorigenesis. Because it is an enzymatic subunit of the human SAGA transcriptional cofactor, USP22 deubiquitylates histone H2A and H2B in the nucleus, thus participating in gene regulation and cell-cycle progression. However, the mechanisms regulating its nuclear translocation have not yet been elucidated. It was here demonstrated that USP22 is imported into the nucleus through a mechanism mediated by nuclear localization signal (NLS). The bipartite NLS sequence KRELELLKHNPKRRKIT (aa152–168), was identified as the functional NLS for its nuclear localization. Furthermore, a short cluster of basic amino acid residues KRRK within this bipartite NLS plays the primary role in nuclear localization and is evolutionarily conserved in USP22 homologues. In the present study, a functional NLS and the minimal sequences required for the active targeting of USP22 to the nucleus were identified. These findings may provide a molecular basis for the mechanism underlying USP22 nuclear trafficking and function.

1. Introduction

The human *USP22* gene is located on chromosome 17 and consists of 14 exons. It shows moderate levels of transcription and expression in various tissues, including those of the heart and skeletal muscle, and it shows weak expression in the lung and liver [1]. Notably, elevated levels of USP22 have been observed in most human cancers, including colorectal cancer [2], lung cancer [3], and breast cancer [4]. Accordingly, down-regulation of USP22 leads to the accumulation of cells in the G1 phase of the cell cycle [5]. In this way, USP22 is a putative cancer stem cell marker, and reducing USP22 expression may be an important strategy for cancer therapy [6].

USP22 is a 525-amino-acid polypeptide consists of the N-terminal zinc finger motif and the C-terminal Cys, Asp (I), His, and Asn/Asp (II) domain with the molecular weight of approximately 60 kDa [1]. In humans, USP22 is present within the SAGA transcriptional co-activator complex and catalyzes the deubiquitylation of histone H2A and H2B, thus participating in gene regulation and cell-cycle progression [7]. USP22 has been shown to be required for the transcription of target genes regulated by the Myc oncoprotein and other sequence-specific activators that require hSAGA activity, including *p21*, *cyclin D2*, and *CAD* [5]. USP22 was also shown to deubiquitinate intracellular protein, including the shelterin protein TRF1 [8], the histone deacetylase Sirt1 [9], and the far upstream element-binding protein 1 FBP1 [10]. These results suggest that USP22 may function in both the nucleus and cytoplasm. However, the mechanisms regulating its nuclear translocation have not been elucidated.

It is well known that most of nuclear proteins are imported into the nucleus through nuclear pore complexes (NPCs) [11]. Relative small proteins (20–40 kDa) can be imported into NPCs by passive diffusion [12]. During this process, large proteins undergo active transport mediated by a nuclear localization signal (NLS) to access the nucleus [13]. Considering that the molecular weight of this protein is approximately 60 kDa, it is here proposed that USP22 nuclear translocation requires NLS induction and undergoes a strictly regulated process.

In order to identify the NLS associated with human USP22, USP22 was fused to enhanced green fluorescent protein (EGFP) and, using a series of deletion constructs, the site of a functional bipartite NLS was determined. Furthermore, mutation of any basic amino acid residues in this region minimizes the nuclear localization of USP22.

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2. Material and methods

2.1. Cloning of USP22

Full-length human USP22 was generated by polymerase chain reaction (PCR) from HeLa cDNA. Primers used for PCR are as follows: forward, 5′AGGATCCATGGTGTCCCGGCCAGAGCCCG-3′; 5′-AGAATTCCTACTCGTATTCCAGGAACTG-3′. The amplified DNA was cloned into PMD 18-T simple vector (TaKaRa Biotechnology, Dalian, China) and verified by direct sequencing. PMD-18-USP22 was digested with *XhoI* and *KpnI* and subcloned into the pEGFP-N1 or pEGFP-C1 (BD Bioscience Clontech, Palo Alto, CA, U.S.A.).

2.2. Deletion and site-directed mutagenesis

In order to facilitate mapping of a potential NLS site, primer pairs were used for amplification of the corresponding USP22 fragments and inserted into the *XhoI*/*KpnI* digested vector pEGFP-N1 and pEGFP-C1, respectively.

Site-directed mutagenesis was carried out on the corresponding deletion constructs using a MutanBEST Kit (Takara Biotechnology, Dalian, China). All mutations were confirmed by DNA direct sequencing.

2.3. Cell culture and transfections

Human cervix adenocarcinoma HeLa cells (obtained from American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a 5% CO_2 and 95% air incubator. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications.

2.4. Visualization of GFP fluorescence

The GFP-plasmid DNA were transiently transfected into HeLa cells with Lipofectamine 2000 according to the manufacturer's directions. At 48 h post-transfection, the cells were fixed with 4% (w/v) formaldehyde in PBS for 10 min at room temperature, then permeabilized with 0.5% Triton X-100 in PBS for 10 min, and the nuclei stained with 0.01 μg/ml DAPI for 15 min. Cells were imaged on a Zeiss Lsm710 confocal microscope, using the appropriate filters for GFP and DAPI. Images were initially imported and analyzed using Zen 2010.

3. Results

3.1. USP22 fused to the GFP was located in the nucleus

Fusion protein comprising green fluorescent protein (GFP) at C-terminus of the USP22 (pEGFP-N1-USP22) or at N-terminus of the USP22 (pEGFP-C1-USP22) were constructed. In HeLa cells transfected with pEGFP-N1-USP22, EGFP fluorescence overlaid with DAPI-stained nuclei demonstrated that USP22 was accumulated in nucleus. Identical results were obtained when cells were transfected with pEGFP-C1-USP22. Cells transfected with the EGFP-empty vector (pEGFP-N1) showed EGFP expression throughout both the cytoplasm and nucleus (Fig. 1). This is probably because the small size of EGFP (27 kDa) can pass through the NPC via passive diffusion. Nuclear localization of EGFP-USP22 was also confirmed in another cell line, HEK293 (data not shown).

3.2. Protein prediction programs were used to identify putative NLSs in USP22

Because USP22 was able to direct GFP to the nucleus, we analyzed the full length sequence of human USP22 by utilizing

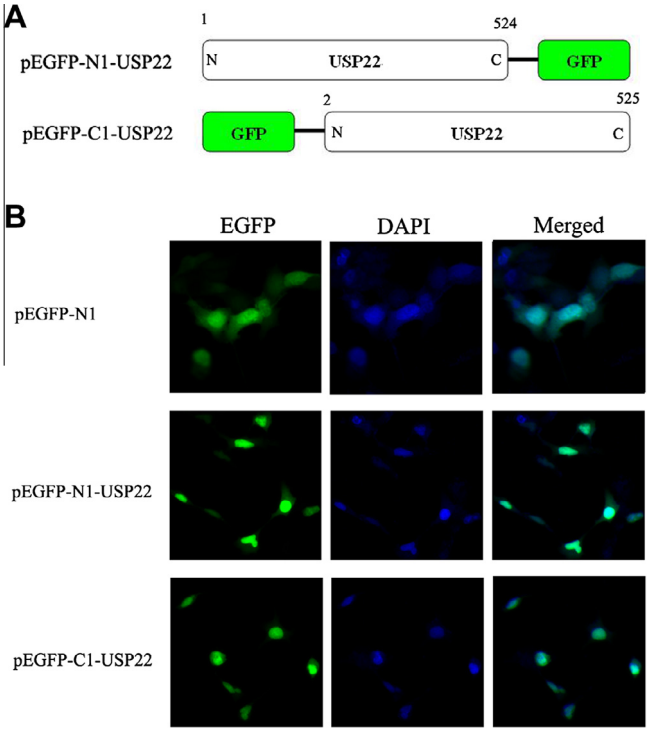

Fig. 1. (A) USP22 fragments fused to GFP. (B) HeLa cells were transiently transfected with pEGFP-N1, pEGFP-N1-USP22, and pEGFP-C1-USP22. From left, representative fluorescent microscopy images of EGFP fluorescence, DAPI-stained nuclear fluorescence and a merged version of these two images.

the protein domain prediction program PSORT II (http://psort.nibb.ac.jp). PSORT analysis revealed two potential NLSs within the USP22 protein. One putative sequence (RKRK), located between amino acids 54 and 57, appeared to be a monopartite NLS. Another putative sequence (KRELELLKHNPKRRKIT), located between amino acids 152 and 168, appeared to be bipartite NLS. However, the PSORT program did not indicate any nuclear export signals (NES) within USP22.

3.3. The N-terminus was required for nuclear localization of USP22

To confirm the database analysis of NLS from PSORT programs and to map which terminal amino acid sequence of USP22 is indeed for its nucleus localization, a series of C-terminal and N-terminal deletion mutants of the USP22-GFP were constructed and transfected into HeLa cells. As shown in Fig. 2, deletion of the N-terminal region 56 amino acids (GFP-USP22Δ57-525) or 154 amino acids (GFP-USP22Δ155-525) did not abolish the accumulation of EGFP in the nucleus. Further deletion of N-terminal 192 amino acids (GFP-USP22Δ193-525) eliminated the ability of the protein to enter the nucleus. However, deletion of the C-terminal region 371 amino acids of USP22 (GFP-USP22Δ1-154) did not direct EGFP to the nucleus. Notably, elongation of the fragment GFP-USP22Δ1-154 to 193 amino acids (GFP-USP22Δ1-193) restored GFP to transport into nucleus. These results suggest the following: (1) the putative NLS (54RKRK57) is not required for USP22 transporting; (2) the predicted bipartite NLS located from amino acid 154 to 168 is a functional NLS that directed USP22 to the nucleus.

3.4. Basic amino acid residues affected the nuclear localization of USP22

A conserved sequence (KRRK) was found within the NLS. This sequence is identified as a short cluster of basic amino acid

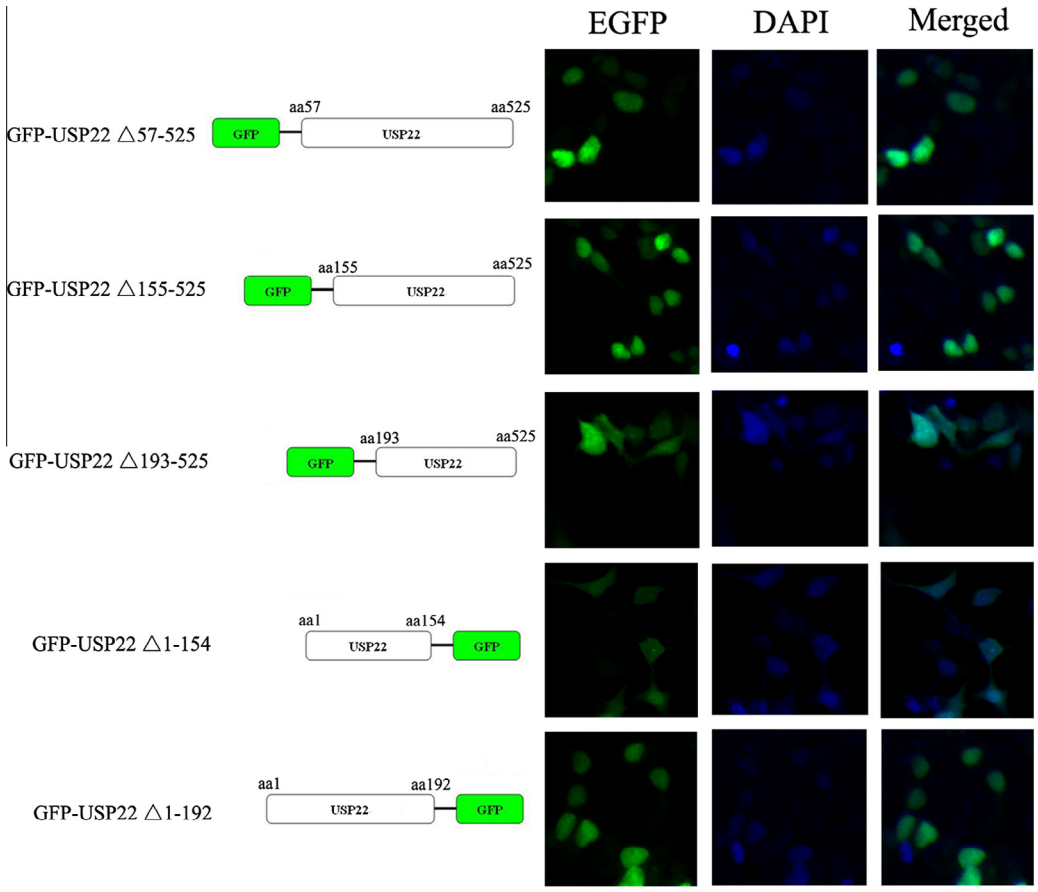

Fig. 2. The N-terminus of USP22 is sufficient for nuclear localization of EGFP. Five fusion proteins were generated and transiently transfected into HeLa cells. Forty-eight hours after transfection, the subcellular localizations of EGFP-tagged proteins were visualized using confocal microscope.

residues for NLS [14]. It is located at amino acids 163–167. To determine whether this cluster was functional and to further determine precisely which amino acid residue is responsible for USP22 nuclear localization, site-directed mutagenesis was performed within full-lengthen USP22. As shown in Fig. 3, mutation of any amino acid residue of 163KRRK167 disrupted the nuclear localization of USP22 in HeLa and led to a diffused cytoplasmic distribution. As the control, mutation of amino acid residue flanking of KRRK did not affect the nuclear location of GFP. These results indicated that all four basic amino acid residues (KRRK) are essential to the nuclear localization of USP22 and the NLS of USP22 seems to be a classical NLS.

3.5. Functional NLS of USP22 was conserved in USP22 homologues

USP22 is an evolutionarily conserved protein that has *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, and *Xenopus* homologues. Alignment of the KRRK motif with USP22 orthologs from other species showed 163KRRK167 to be highly conserved among the species examined (Fig. 4). However, USP22 was found to lack homology to other known USP members, including USP2, USP7, and USP36.

4. Discussion

USP22 is the subunit of the human SAGA transcriptional co-activator complex. USP22 catalyzes the deubiquitylation of histone H2A and H2B in the nucleus, thus participating in gene regulation and cell-cycle progression [7]. Recently, USP22 nuclear expression has been proved to be significantly associated with tumor progression [9]. All these findings indicate that the appropriate nuclear location of USP22 is critical to its physiological function. However, the mechanism by which USP22 was transported into the nucleus was not identified.

It is well known that exchange of proteins between the cytoplasm and the nucleus is dependent on the nuclear pore complex (NPC) [15]. Proteins of different sizes may pass through the NPC in different ways. Relatively small molecules (less than 40 kDa) can be imported into NPCs by passive diffusion, and large proteins are imported by active translocation, which is mediated by reciprocal recognition and binding between importin receptor and NLS [13]. Considering the relatively large molecular weight (approximately 60 kDa), USP22 could not theoretically enter the nucleus passively. Characterization of the NLS is required for revealing the mechanism underlying USP22 nuclear translocation.

In this study, a functional classical NLS was identified in the N-terminus of USP22. Four basic amino acid residues (KRRK) indispensable to USP22 import were identified. This functional KRRK was highly conserved in the USP22 proteins of other species, but it lacks homology to other known USP proteins.

USP22 is a member of deubiquitinating enzyme family. It contains a carboxy-terminal ubiquitin hydrolase domain that exerts hydrolase activity [1]. In addition, USP22 contains an amino-terminal zinc finger motif that may mediate the association of itself with other proteins [16]. Using the protein domain prediction program PSORT II, two putative NLS were identified within USP22. One is a monopartite signal (KKRK) located at amino acids 54–57. The other is a bipartite sequence (KRELELLKHNPKRRKIT) located at amino acids 152–168. Earlier reports have indicated that proteins may contain two or more functional NLSs [17], so an attempt was made to determine the functionality of the two putative NLSs.

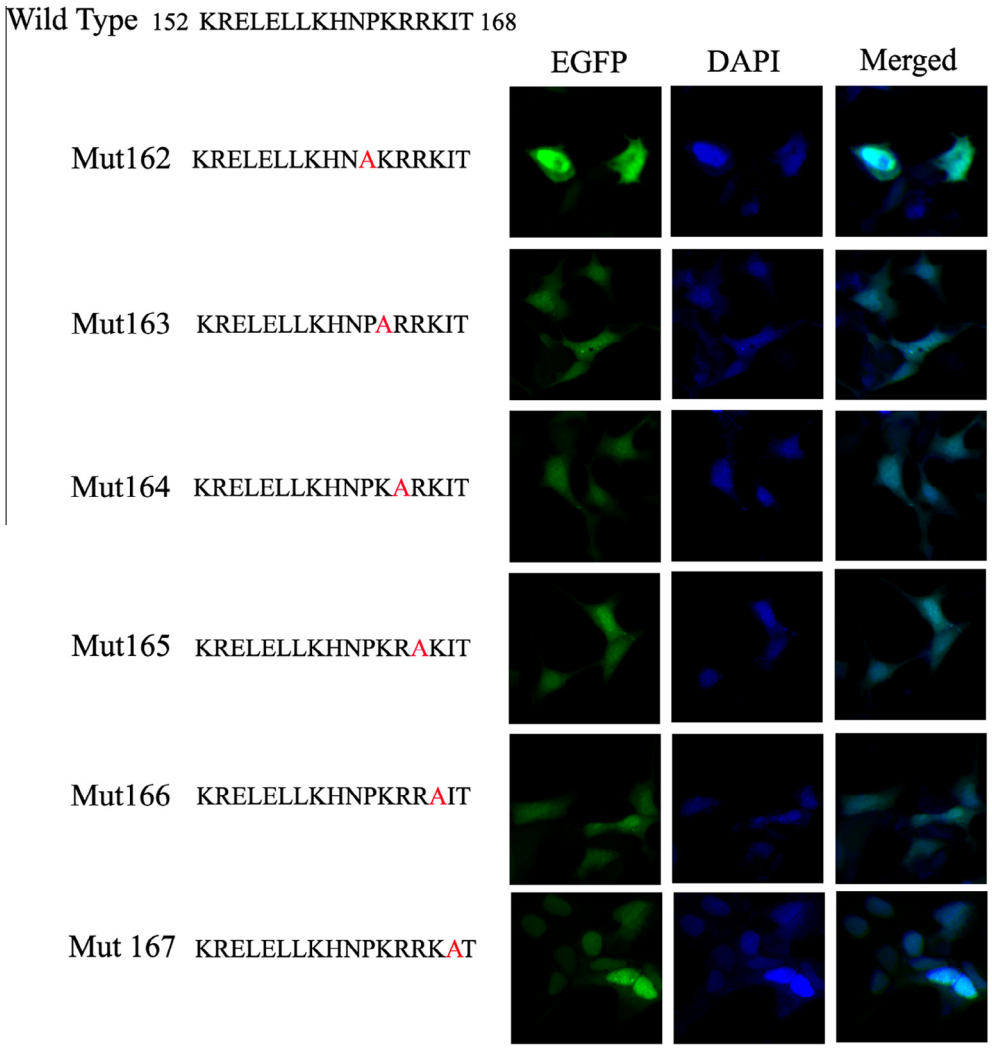

Fig. 3. Basic residues within each of the NLS are required for nuclear localization of GFP.

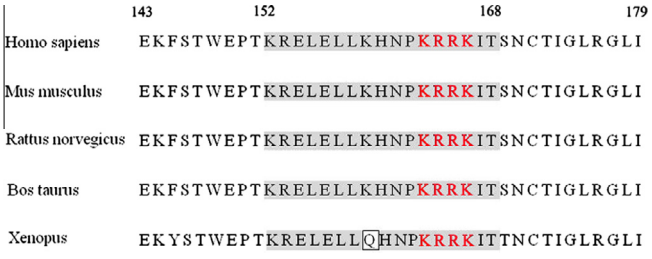

Fig. 4. The functional NLS identified from residues 152 to 168 is highly conserved. A sequence alignment of the functional NLS identified within human USP22 with that of *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, and *Xenopus*. Amino acids conserved within the functional NLS are shaded gray and numbers of residues corresponding to the human sequence are also shown.

Although KKRK has been identified as a conserved NLS in several reports [18]. This motif was not found to be responsible for the nuclear localization of USP22. Alternatively, the functional NLS was found embedded between the carboxy-terminal ubiquitin hydrolase domain and amino-terminal zinc finger motif of USP22.

NLS, recognized by import receptors, is a part of an amino acid sequence that exists randomly in N-terminal, middle, or C-terminal of proteins. The classic NLS is typically made of lysines (K) or arginines (R), which are organized as a single-stretch monopartite NLS: (K/R)4–6 [19] or as a bipartite NLS in which there are two small clusters typically separated by 10–12 amino acids (K/R)2X10–12(KR)3 [20]. During nuclear import, NLSs are typically recognized in the cytoplasm by a heterodimeric complex consisting of importins α and β. The NLS protein-receptor complex docks to the nuclear pore complex via importin β and is subsequently translocated through the pore using an energy-dependent mechanism [21]. Although amino acid residues KRELELLKHNPKRRKIT were predicted to be the bipartite NLS for USP22, the key amino acids were found to be a short cluster, KRRK. This cluster is required for USP22 translocation.

As the classical NLS, KRRK is embedded within several proteins, including p100 [14], Maf1 [22], and pUL48 [23]. Mutation of this motif disrupted the movement of these proteins to the nucleus. However, KRRK is not indispensable to all protein importing especially for proteins with multi NLS. For example, disruption of KRRK did not abolish retinitis pigmentosa (RP2) binding to importin receptor [24]. These alterations may be attributed to the fact that the regulation of nuclear transport mechanisms is complex. During translocation process, the NLS in the cargo can be masked by a second macromolecule, affecting the importation of the cargo protein. Under other conditions, post-translational modifications, such as phosphorylation within or proximal to the NLS may change the interaction between NLSs and receptors [25].

This functional NLS, KRRK, was found to be highly conserved in the USP22 protein of other species, including *M. musculus*, *R. norvegicus*, *B. taurus*, and *Xenopus*, suggesting it has a critical role in USP22 nuclear translocation. However, the KRRK motif is not conserved in USP2, USP7, or USP36, which are the other members of the USP family. In fact, although USP22 contained highly conserved Cys, Asp (I), His, and Asn/Asp (II) domains characteristic of the ubiquitin-specific processing proteases, the fact that the regulation it showed low protein homology to other known USP proteins, such as USP2, USP7, and USP36 [1]. These data suggest that USP22 may function in a specialized manner in the DUB family, although the exact mechanism is undefined.

In eukaryotic cells, the movement of molecules into the nucleus via the nuclear pore complex is a crucial event, and mislocation of specific proteins has been linked to numerous cancers and developmental disorders. For example, deregulation of NF-κB localization has been found to be associated with breast, ovarian, colon, pancreatic, and thyroid cancers and with Hodgkin's lymphoma [26]. The regulation of the nuclear translocation has been a novel potential target for several cancer therapies [27]. Regarding USP22, its nuclear accumulation has been linked to tumor progression and unfavorable clinical outcome [28]. However, no direct evidence has shown that deregulation of USP22 translocation has any functional consequences. In light of this, further experiments on USP22 translocation mechanisms are required.

The results of the present study indicate the existence of a functional NLS in USP22. This NLS is essential for moving USP22 to the nucleus. These findings provide a molecular basis for the mechanism underlying USP22 nuclear trafficking and function.

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: J.J.X., W.D.L. Performed the experiments: J.J.X., Y.Q.W. Analyzed the data: G.Z. Contributed reagents/materials/analysis tools: J.Y.L. Wrote the paper: J.J.X., W.D.L.

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