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Note

Tryptophans 286 and 288 in the C-terminal Region of Protein B23.1 are Important for Its Nucleolar Localization

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Nucleolar protein B23 can shuttle between the nucleolus and cytoplasm. However, the mechanism involved in the protein moving and staying in the nucleolus is not fully understood. To identify the nucleolar localization signal sequence of protein B23, we examined the subnuclear location of B23.1 mutant proteins fused with green fluorescent protein in HeLa cells. The results suggested that the two C-terminal tryptophan residues (Trp-286 and Trp-288) of protein B23.1 were important in this phenomenon.

Key words: protein B23; nucleolar localization; nucleolar protein; tryptophan

Protein B23 (also known as nucleophosmin/NPM, NO38, or numatrin), one of the abundant nucleolar proteins, seems to be involved in ribosome biogenesis. Protein B23 shuttles between the nucleolus and cytoplasm,[1] but little is known about the mechanisms by which these proteins reach or stay in the nucleolus. Studies of the nucleolar accumulation of viral proteins such as HIV-1 Rev[2] and Tat[3] proteins and HTLV-1 Rex[2] protein have suggested that, like nuclear transport, nucleolar transfer is mediated by short amino acid sequences, acting as nucleolar localization signals. Deletion experiments showed that a domain of about 24 amino acid residues near the C-terminus of the *Xenopus laevis* nucleolar protein NO38, which corresponds to B23 of mammals, is essential for nucleolar localization.[4] However, fusion of the domain to a reporter protein does not inevitably result in the chimeric protein moving to the nucleolus.[4] Rat protein B23 exists as two isoforms, designated B23.1 and B23.2, which are polypeptides of 292 and 257 amino acid residues, respectively.[5] The N-terminal 255 residues are identical in the two isoforms, but the C-terminal ends of the two proteins are different. The two forms are generated from a single gene *via* alternative splicing of 3′ exons at the mRNA level.[5] Protein B23.1 almost always found only in the nucleolus, and B23.2 is found in the nucleoplasm and cytoplasm fractions, which suggests that the C-terminal 35 amino acids of B23.1 may be essential for the nucleolar localization.[6] In this study, we identified a more detailed site in the C-terminal region of B23.1 that contributes to the phenomenon.

Green fluorescent protein (GFP) was fused to wild-type rat B23 isoforms or C-terminal deletion mutants of B23.1 (Fig. 1(A)). All GFP-B23 fusion proteins were expressed in HeLa cells and the expressed proteins were checked by immunoprecipitation of the nuclear extracts 48 h after transfection (Fig. 1(B)). Expression of all GFP-B23 constructs resulted in the synthesis of fusion proteins of the predicted lengths and at approximately the same concentrations. Some proteolytic fragments of the GFP-B23 fusion proteins were recognized by the anti-B23.1 antibody. The expression of GFP-B23 fusion proteins in HeLa cells was observed by fluorescence microscopy (Fig. 1(C)). For control experiments, HeLa cells were transfected by the vector pEGFP-C1. The empty vector resulted in a diffuse distribution of GFP throughout the nucleus and cytoplasm, but all GFP-B23 fusion proteins were found only in the nucleus. Nucleolar proteins such as NO38 and nucleoplasmin have two copies of a putative nuclear accumulation sequence.[7,8] Such sequence was found in rat protein B23 at positions 152–157 and 190–196.[9] B23.1, B23.2, and all other mutants had that sequence, so all GFP-B23 fusion proteins were retained in the nucleus. However, the subnuclear distribution of the mutant proteins was depended on the protein. GFP fused with B23.1, ΔC3, and ΔC6 were found in the nucleolus. In contrast, GFP fused with B23.2 and ΔC9 were found in both the nucleolus and nucleoplasm. In other words, B23.2 and ΔC9 failed to accumulate in the nucleolus, suggesting that the C-terminal 9-residue segment of protein B23.1 was essential for movement to the nucleolus.

The C-terminal region of rat B23.1 has five aromatic residues (Fig. 2). The aromatic residues, espe-

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Abbreviations: GFP, green fluorescent protein

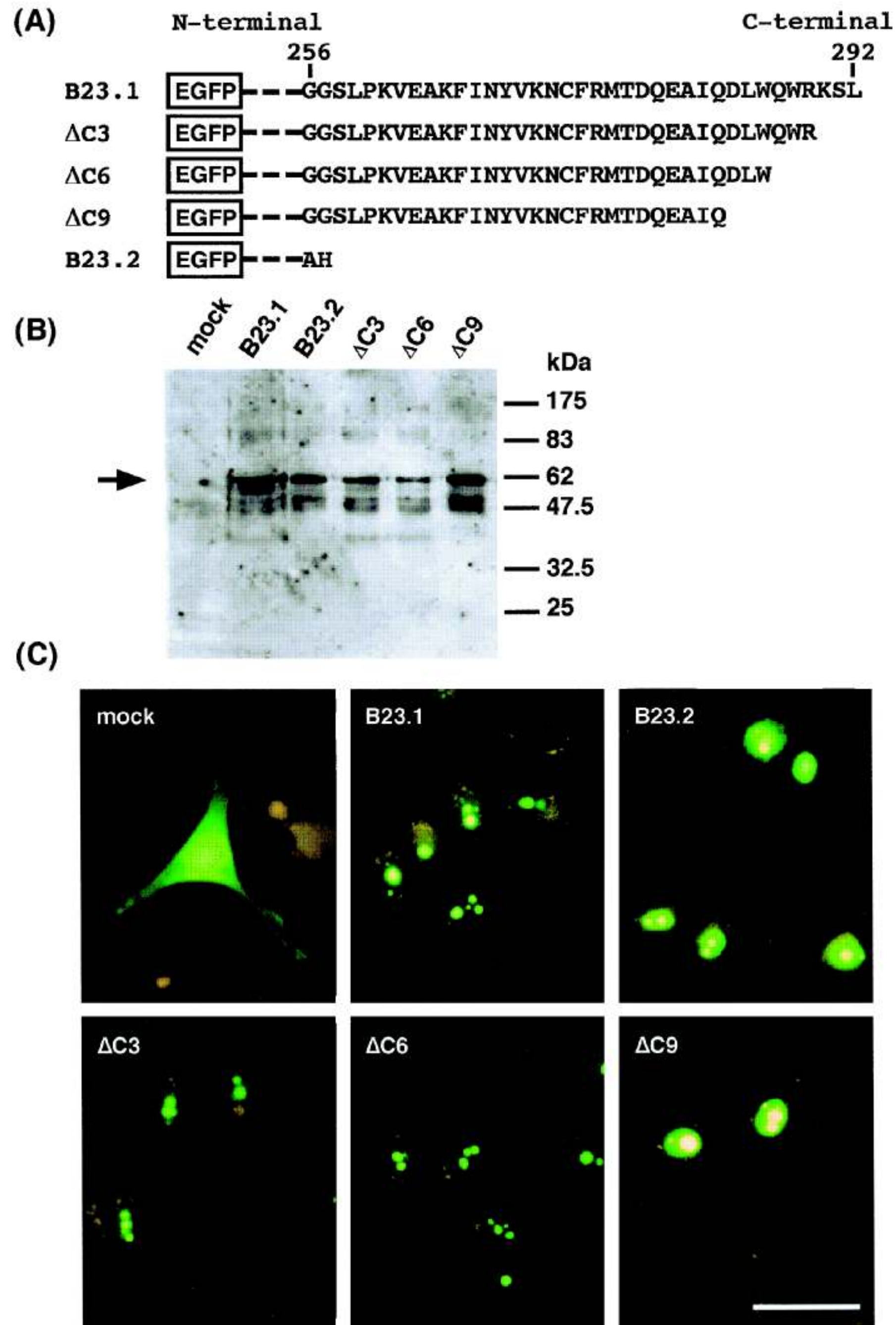

Fig. 1. Deletion Scheme (A), Immunoprecipitation (B), and Expression of GFP-B23.1 Mutants (C).

(A) C-terminal amino acid sequences of GFP-B23 isoforms and deletion mutants. All GFP fusion proteins were constructed by PCR cloning into pEGFP-C1 (Clontech Laboratories, Inc.). In all, GFP was fused to the N-terminus of the proteins. The PCR primers for B23.1, B23.2, and deletion mutants of B23.1 were B1N (5′-GCCTCGGATCCATGGAAGACTCGATG-3′) and B1C (5′-GG-ATCCTTAAAGAGACTTCCTCCACT-3′) for full-length B23.1; B2N (5′-AAGCTTCGATGGAAGATCGATGGA-3′) and B2C (5′-AAGCTTCCTCCACTGCCAGAGATCTT-3′) for ΔC3; B1N and B3C (5′-AAGCTTCCAGAGATCTTGAATAGCCT-3′) for ΔC6; B1N and B4C (5′-AAGCTTCCGAATAGCCTCCTGGTCAG-3′) for ΔC9; and B2N and B5C (5′-GGATCCTCAATGCGCTTT-TTCTATAC-3′) for B23.2. All fusion constructs were sequenced and found to be accurate copies of the corresponding genes.[9] (B) Target DNA was electroporated in a Bio-Rad electroporator at 250 V and 950 mF. Nuclear extracts were prepared from 1×10^7 cells 48 h after transfection and treated to immunoprecipitation with Living Colors Full-Length A.v. polyclonal antibodies (Clontech Labs.) with IMMUNOcatcher (CytoSignal, Inc.). The pellet was used in 12.5% SDS-PAGE[16] and blotted onto a membrane. The proteins were detected with monoclonal anti-rat B23.1 antibody[17] diluted 1:1000 and peroxidase-conjugated goat anti-mouse IgG diluted 1:3000. The presence of immunobands was verified with a western blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.). Molecular weight standards are indicated on the right in kDa. (C) The expression constructs were used to transfect HeLa cells for a time by electroporation. Cells were then seeded onto glass coverslips mounted on the bottom of a dish and allowed to grow for 48 h. Cells were fixed with 10% formaldehyde in phosphate buffered saline (PBS) for 30 min and mounted in 90% glycerol. The location of GFP-B23 fusion proteins was then observed with a fluorescence microscope (Olympus, Tokyo). Bar, 25 μm.

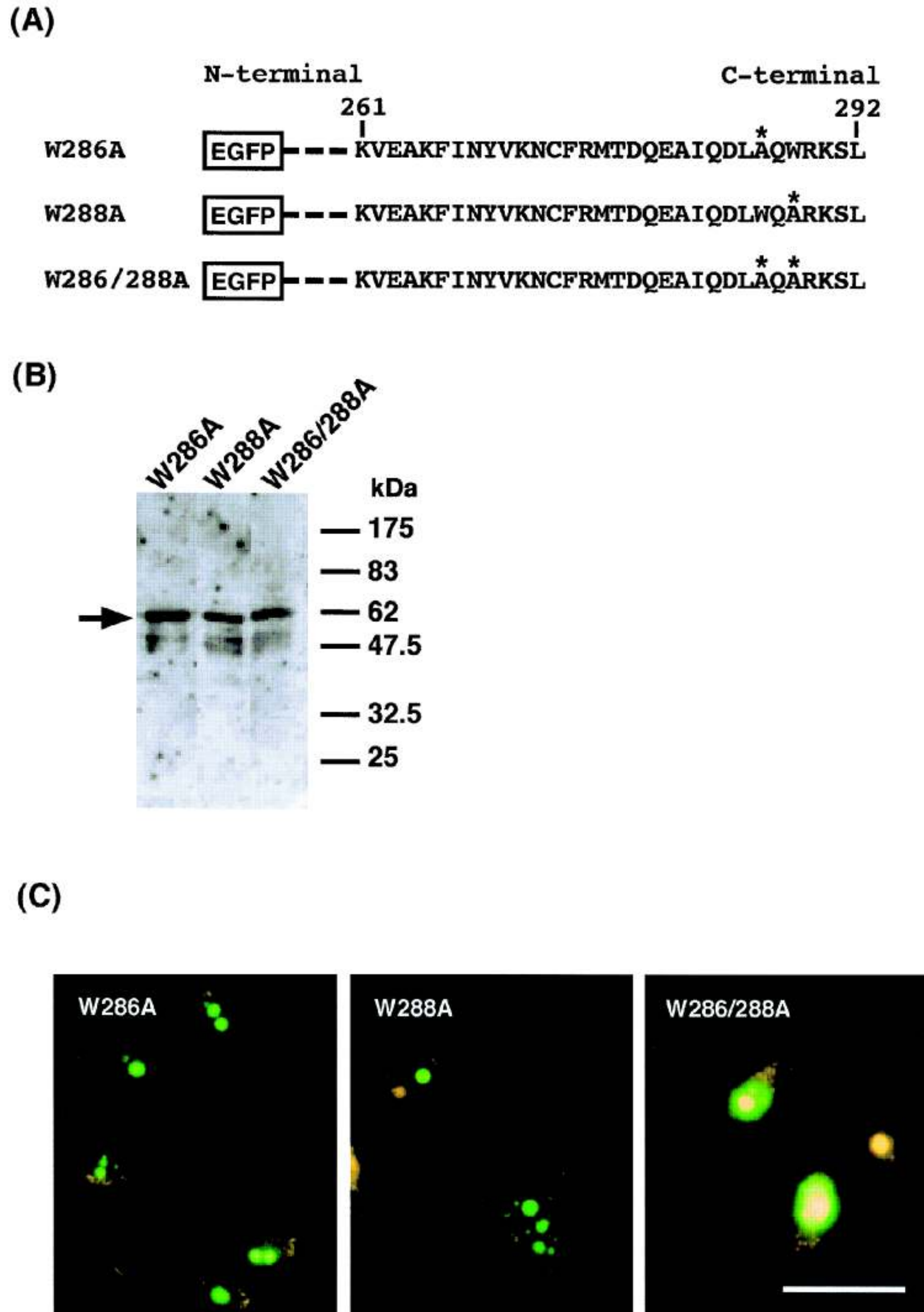

Fig. 3. Point Mutation Scheme (A), Immunoprecipitation (B), and Expression of GFP-B23.1 Mutants (C).

C-terminal amino acid sequences of GFP mutant proteins. An asterisk above a residue indicates alanine substituted for tryptophan. In all, GFP was fused to the N-terminus of proteins. The PCR primers for point mutants of GFP-B23.1 were B2N and B6C (5′-AAGCTTTTAAAGAGACTTCCTCCACTGCGCGAGAT-3′) for W286A; B2N and B7C (5′-AAGCTTTTAAAGAGACTTCC-TCGCCTGCCA-3′) for W288A; and B2N and B8C (5′-AAGCTTTTAAAGAGACTTCCTCGCCTGCGCGAGAT-3′) for W286/288A. Western blotting (B) and fluorescence microscopy (C) of GFP-B23 mutant proteins were done by the procedures in the legend of Fig. 1. Bar, 25 μm.

cially the two tryptophans, were highly conserved in the analogous proteins sea-urchin mitosis-apparatus protein p62,[10] starfish nucleolar protein ANO39,[11] and *Xenopus laevis* NO38.[7] The two tryptophans were in the C-terminal 9-residue segment of protein B23.1. We fused GFP with the point mutant protein in the C-terminal region of protein B23.1 that has alanine instead of tryptophan, and expressed the fusion protein in HeLa cells (Fig. 3). The expression of GFP fusion point mutants was checked by immunoprecipitation (Fig. 3(B)). The single tryptophan mutants W286A and W288A accumulated in the

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rat B23.1  261 KVEAKFINYVKNCFRMTDQEAIQDLWQW 288
p62        375 KKEEKFKNFVKSKFHLSEGKKIQELWGW 402
ANO39      312 KKEEKFKNYVRSAFHISEAKKLQDLWGW 339
NO38       261 KVEVKFANYVKNCFRTDSQKVIQDLWKW 288
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Fig. 2. Amino Acid Sequence of a Segment of Rat Protein B23.1.[19] Compared with Those of Analogous Proteins: Sea Urchin p62,[10] Starfish ANO39,[11] and *Xenopus laevis* NO38.[7]

Identical residues were lined by vertical lines. The two tryptophans of the C-terminal region of B23.1, conserved in the analogous proteins, are indicated by an asterisk.

nucleolus (Fig. 3(C)), as did the with wild-type GFP-B23.1 fusion protein. However, when both Trp-286 and Trp-288 were replaced with alanine, the GFP mutant protein W286/288A did not accumulate in the nucleolus. These results suggest that tryptophans 288 and 286 in protein B23.1 were important for its nucleolar localization.

The two isoforms of protein B23 have intrinsic ribonuclease activity, but the shorter form has less activity than the longer form, suggesting that the C-terminal end of protein B23.1 is critical for substrate binding.[12,13] Although the HIV-1 Tat protein differs from protein B23.1 in the sequence needed for nucleolar localization, the domain is in the RNA-binding region.[3,14,15] Therefore, tryptophans 286 and 288 may allow the access of protein B23.1 to preribosomal RNA in the nucleolus. Using amphibian oocytes, Peculis and Gall found that deletion of the C-terminal 11 amino acids of *Xenopus laevis* NO38 containing the two tryptophans does not alter nucleolar accumulation.[4] Therefore, the need for two C-terminal tryptophans for the nucleolar localization of protein B23.1 might be specific to mammalian cells.

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