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Restoration of nuclear-import failure caused by triple A syndrome and oxidative stress

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ABSTRACT

Triple A syndrome is an autosomal recessive neurological disease, mimicking motor neuron disease, and is caused by mutant ALADIN, a nuclear-pore complex component. We recently discovered that the pathogenesis involved impaired nuclear import of DNA repair proteins, including DNA ligase I and the cerebellar ataxia causative protein aprataxin. Such impairment was overcome by fusing classical nuclear localization signal (NLS) and 137-aa downstream sequence of XRCC1, designated stretched NLS (stNLS). We report here that the minimum essential sequence of stNLS (mstNLS) is residues 239–276, downsized by more than 100 aa. mstNLS enabled efficient nuclear import of DNA repair proteins in patient fibroblasts, functioned under oxidative stress, and reduced oxidative-stress-induced cell death, more effectively than stNLS. The stress-tolerability of mstNLS was also exerted in control fibroblasts and neuroblastoma cells. These findings may help develop treatments for currently intractable triple A syndrome and other oxidative-stress-related neurological diseases, and contribute to nuclear compartmentalization study.

Triple A syndrome is an autosomal recessive neuroendocrinological disease [1]. In children, triple A syndrome is clinically characterized by adrenocorticotropic hormone-resistant adrenal failure, achalasia, and alacrima. By contrast, in adults this syndrome is primarily associated with a motor neuron disease-like phenotype, showing progressive involvement of upper and lower motor neurons [2]. Causative mutations for both the childhood and adult types of triple A syndrome have been identified in a gene that encodes ALADIN, a component of nuclear-pore complex (NPC). Vertebrate NPC comprising 30–40 different proteins contains an aqueous channel that allows passive diffusion of molecules smaller than 40–60 kDa, but restricts trafficking of larger molecules between the cytoplasm and nucleus [3]. To overcome this barrier, macromolecular proteins have specific signals that allow them to access the nucleocytoplasmic transport machinery of cells. In a major nuclear-import pathway, proteins containing nuclear localization signals (NLS) attach to soluble carriers of the *karyopherin* family [4].

We recently showed that the primary defect caused by disease-associated mutant ALADIN is the selective failure to import the DNA repair proteins aprataxin (APTX) and DNA ligase I into the nucleus, resulting in increased DNA damage and consequent cell death under oxidative stress. APTX is a causative protein for autosomal recessive spinocerebellar degeneration that affects motor neurons in addition to cerebellar neurons [5,6]. In contrast to these proteins, ALADIN mutation does not affect the import of several other proteins with classical NLS, such as X-ray repair cross-complementing 1 (XRCC1), a scaffold DNA repair protein associated with susceptibility to several types of cancer [7]. We then identified stretched NLS (stNLS) containing NLS of XRCC1 and 137 amino acids (aa) downstream, which enabled APTX and DNA ligase I to efficiently enter the nucleus in fibroblasts from a patient with an ALADIN gene mutation. However, a serious concern is that stNLS, consisting of 146 aa (molecular weight >18 kDa), is a relatively large molecule that might affect functions of fused proteins when used as a tag. In the present study, we identified the minimum sequence of stNLS (mstNLS) that retains efficient nuclear localization and used it as a tag for a therapeutic option in triple A syndrome.

Materials and methods

Vector construction and transfection. To construct GFP–GFP (GFPx2)-vector, EcoRI/BsrGI fragment containing GFP cDNA was further inserted into EcoRI/SalI sites of the modified GFP-N1 vector (delMet), which lacks the first ATG for GFP, thereby preventing the start of translation from the second GFP. Truncated XRCC1 cDNA (encoding codons 239–266, 239–269, 239–276, 239–289, 270–276, or 239–403) was inserted into EcoRI/SalI sites of GFPx2

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vector. Vectors for GFP-APTX, -ligase I, and -wild-type (wt) ALADIN were already constructed as described previously [5,8]. GFP-SOD1 was constructed by PCR amplification of full-length cDNAs and cloning into pEGFP-C1. mstNLS-fused GFP-APTX, -ligase I, and -SOD1 vectors were generated by insertion of mstNLS into the upstream of GFP-fused protein in each vector. GFP-SMN1 and GFP-XPA were generously donated by Dr. Matera and Dr. Vermeulen, respectively. All vectors were sequenced to eliminate possible PCR or ligation errors. Cells were maintained in a 5% CO_2 humid atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The EGFP vectors were transfected into mouse NIH3T3 cells, human SK-N-MC neuroblastoma cells, and primary skin fibroblasts from a patient with Ile482Ser mutation in the ALADIN gene [5] and into fibroblast from an age- and sex-matched control with the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Amounts of GFP vectors for transfection were adjusted by adding LacZ vector in equal molar concentrations. Forty-eight hours after transfection, the cells were subjected to fluorescent microscopic analysis. GFP proteins expressed in live cells were quantified with the use of a fluorescence microscope (DMIRB, Leica, Germany), which allowed us to rapidly analyze the number of cells without subcellular fixation-related artifacts [9].

Nuclear accumulation assay. The nucleus and the entire cell were delineated by superimposing a GFP image with a Hoechst 33258-stained image and a phase contrast image, respectively [5]. For semi-quantitative analysis, cells were scored into the following categories: N, green fluorescence was almost exclusively detected in the nucleus; N > C, green fluorescent intensity in the nucleus was stronger than that in the cytoplasm; N ⩽ C, green fluorescent intensity in the nucleus was equal to or lower than that in the cytoplasm. Under our experimental conditions, no cells had GFP proteins exclusively in the cytoplasm. Data were obtained from at least 300 cells in three independent experiments, and the results were expressed as percentages.

Cell survival assay for complementation of BSO sensitivity. Patient fibroblasts 32 h after transfection with GFP-wtALADIN and mstNLS and/or stNLS protein expression vectors, and control LacZ vector were treated with 0–1 mM L-buthionine-(*S*,*R*)-sulfoximine (BSO), a glutathione-depleting agent that accumulates endogenous

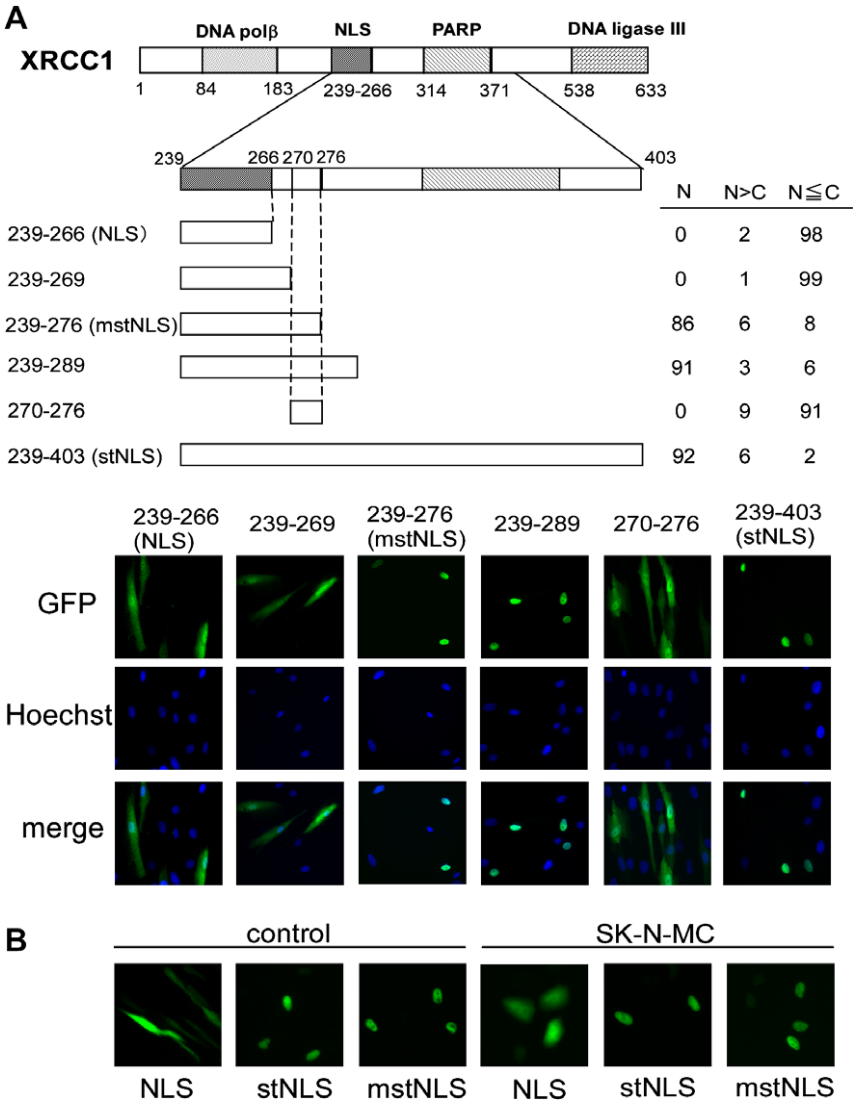

Fig. 1. Identification of mstNLS. (A) XRCC1, a 633-amino-acid protein, has a nuclear localization signal (NLS) and binding sites for various DNA repair proteins, including DNA polymerase β (polβ), poly (ADP-ribose) polymerase (PARP), and DNA ligase III (upper panel). Fluorescence microscopic analyses of triple A patient fibroblasts transfected with GFPx2-constructs expressing various XRCC1 fragments showed distinct nuclear (N) and cytoplasmic (C) distributions of fluorescence (lower panels). Cells were scored as described in Materials and methods, and mean values from five independent experiments are expressed as percentages. Residues 239–276 (msNLS) were found to be essential for nuclear localization. (B) The identified mstNLS also functioned in control fibroblasts and SK-N-MC neuroblastoma cells.