Gene 519 (2013) 217–221

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Nijmegen breakage syndrome: The clearance pathway for mutant nibrin protein is allele specific

Bastian Salewsky [1], Petra Wessendorf [1], Daniel Hirsch, Harald Krenzlin, Martin Digweed *

Charité-Universitätsmedizin Berlin, Institute of Medical and Human Genetics, Augustenburger Platz 1, 13353 Berlin, Germany

ARTICLE INFO

Article history:
Accepted 8 February 2013
Available online 28 February 2013

Keywords:
DNA repair
Proteasome
Lysosome
Microautophagy

ABSTRACT

The autosomal recessive disorder Nijmegen breakage syndrome (NBS) is caused by mutations in the *NBN* gene which codes for the protein nibrin (NBS1; p95). In the majority of cases, a 5 bp deletion, a founder mutation, leads to a hypomorphic 70 kD protein, p70-nibrin, after alternative initiation of translation. Protein levels are of relevance for the clinical course of the disease, particularly with regard to malignancy. Here, mechanisms and efficiency of mutant protein clearance were examined in order to establish whether these have an impact on nibrin abundance. Cell lines from NBS patients and retroviral transductants were treated with proteasome and lysosome inhibitors and examined by semi-quantitative immunoblotting for p70-nibrin and p95-nibrin levels. The results show that p70-nibrin is degraded by the proteasome with varying efficiency in cell lines from different NBS patients leading to lower or higher steady state levels of this partially active protein fragment. In contrast, a previously described *NBN* missense mutation, which disturbs protein folding due to the substitution of a critical arginine by tryptophan, was found to be cleared by lysosomal microautophagy leading also to lower cellular levels. The data show that truncated nibrin and misfolded nibrin have different clearance pathways.

1. Introduction

Cardinal symptoms of Nijmegen breakage syndrome (NBS; OMIM 251260) are characteristic facial features, microcephaly, humoral and cellular immunodeficiency, radiosensitivity and lymphoreticular malignancies (Chrzanowska et al., 2012). Approximately 40%–50% of NBS patients develop a lymphoma before the age of 20. Cells from NBS patients have a high rate of spontaneous chromosome breakage. In lymphocytes, clonal and non-clonal rearrangements involving the T-cell receptor and immunoglobulin genes are characteristic. Occasionally, so called telomere fusions are also observed. Cellular sensitivity towards ionizing radiation is extreme, particularly the failure to arrest DNA synthesis after irradiation.

The gene underlying NBS is *NBN* whose product, nibrin (p95), is part of a trimeric complex together with MRE11 and RAD50 (the MRN complex). This complex is evolutionarily highly conserved, and in yeast consists of the proteins Mre11, Rad50 and Xrs2, whereby Xrs2 represents the functional orthologue of nibrin. In yeast, and also in human cells, the MRN complex is involved in DNA double strand break (DSB) repair by both homologous recombination and non-homologous end joining, the two principle DSB repair pathways of mammalian cells. Nibrin is apparently a sensor of DSBs as it is required for relocation of the complex to the sites of DSBs after irradiation (Carney et al., 1998). In addition to a direct role in DNA repair, the complex is also involved in the activation of ATM, the protein mutated in Ataxia telangiectasia, and thus in the triggering of cell cycle checkpoints (Lee and Paull, 2004). The vast majority of NBS patients is homozygous for a founder mutation, c.657_661del5 (p.K219fsX19), in exon 6 of the gene (Varon et al., 1998). Alternative translation of the *NBN* mRNA leads to a carboxyterminal fragment, p70-nibrin, with residual function (Demuth et al., 2004; Difilippantonio et al., 2005; Maser et al., 2001). Null mutation of *Nbn* in the mouse is lethal (Dumon-Jones et al., 2003).

ATM and nibrin are also involved in the processing of the DSBs that occur during the maturation of immunocompetent cells. ATM is involved in V(D)J recombination in T-cells and B-cells. While nibrin is not involved in this process, it is clearly involved in immunoglobulin class switching (Kracker et al., 2005), explaining the deficiencies in serum IgG and IgA observed in NBS patients (van Engelen et al., 2001).

The major life threatening symptom of Nijmegen breakage syndrome is the development of a lymphoma in early childhood. The roles of the underlying gene in the repair of DSBs and in immune gene rearrangements are thought to explain this early and specific malignancy. In heterozygotes, increased cancer occurrence has also been reported although here there is no obvious organ specificity and occurrence is much later in life (Seemanova et al., 2007). We

Abbreviations: BRCT, BRCA1 C-Terminus; DSB, DNA double-strand break; LCL, lymphoblastoid cell line; *NBN*, nibrin; NBS, Nijmegen breakage syndrome; PMN, piecemeal microautophagy of the nucleus; RT-PCR, reverse transcription polymerase chain reaction.

* Corresponding author. Tel.: +49 30 450 566 016.
E-mail address: martin.digweed@charite.de (M. Digweed).

[1] These authors contributed equally to the work reported here.

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.02.033

have previously argued that the retention of the wild type allele in the tumors of heterozygotes, and in an appropriate mouse model, indicates that *NBN* is unlikely to be a classical tumor suppressor gene requiring two hits in order to promote tumorigenesis.

Particularly low amounts of p70-nibrin in lymphoblastoid B-cell lines correlate with B-cell lymphoma development in the corresponding patients (Kruger et al., 2007). Furthermore, the missense *NBN* mutation, c.643C>T (p.Arg215Trp), is associated with low levels of p95-nibrin-Trp^{215} in cell lines and with cancer in heterozygous carriers (Seemanova et al., 2006; Bogdanova et al., 2008). Therefore, the picture emerging is one of a haploinsufficient tumor suppressor: even loss of one allele increases cancer risk (Demuth and Digweed, 2007). This is in line with the concept of DNA repair as a primary cancer avoidance pathway, in which repair enzyme levels are critical for lifetime cancer risk (Bartek et al., 2007).

Since p70-nibrin variation is not determined at the level of transcription, and *in vitro* inhibition of the proteasome increases cellular levels of p70-nibrin, we hypothesized that variation in proteasome activity might govern p70-nibrin levels (Lins et al., 2009). Here we present evidence that this is indeed the case. In contrast, the lower levels of a full length nibrin protein due to the mutation c.643C>T (p.Arg215Trp) is not due to the proteasome but, rather, lysosomal microautophagy.

2. Materials and methods

2.1. Cell lines and cultivation

Lymphoblastoid cell lines (LCLs) were grown in RPMI with 10% fetal calf serum and antibiotics at 37 °C, 5% CO_2. Logarithmic growth of cells was ensured by regular cell counting and subculture. 94P0197 is a control cell line, 94P0111 is heterozygous for the *NBN* founder mutation c.657_661del5 and 04P0014 is the cell line from a patient compound heterozygous for the c.657_661del5 and c.643C>T (p.Arg215Trp) mutations (Seemanova et al., 2006). The immortalized human NBS fibroblast cell lines GM07166VA7 (Komatsu et al., 1996) and NBS-1LBI (Kraakman-van der Zwet et al., 1999), homozygous for the *NBN* founder mutation c.657_661del5, were cultured in Dulbecco's minimal essential medium with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO_2.

2.2. Retroviral transduction

The retroviral vector pLXIN-*NBN* carrying the wild type *NBN* cDNA has been described previously (Cerosaletti et al., 2000). Site directed mutagenesis was used to derive a vector containing the c.643C>T *NBN* mutation. pLXIN-*NBN*-C^{643} or pLXIN-*NBN*-T^{643} plasmid DNAs were transfected into Phoenix Eco cells for packaging. Viral supernatants were collected 48 h after transfection and transduction of GM07166VA7 cells was carried out by exposure to viral supernatants at intermediate confluence in 6-well plates. Three rounds of transduction were performed in the presence of 7.5 μg/ml protamine (Roche). Two days after the final transduction, G418 was added to the cultures to 400 μg/ml. Stable transductants were isolated 10 days later, expanded and checked for vector and *NBN* sequences.

2.3. RT-PCR and single nucleotide primer extension

RNA was extracted according to standard procedures (Trizol) from cultured control 94P0197, and NBS 04P0014, LCLs. RNA preparations were treated with DNAseI to remove contaminating genomic DNA. For the first-strand cDNA synthesis, 2 μg RNA of each sample, MMLV reverse transcriptase and random hexamer primers were used. The subsequent RT-PCR reaction used 2 μl of the cDNA product and specific *NBN* primers for amplification of exon 6 (F-primer: 5′-CAGATAGTCACT CCGTTTACAA; R-primer 5′-CATGATCACTGGGCAGGTC). PCR products were used in single nucleotide primer extension reactions with forward (5′-AAAATGTTGATCTGTCAGGA) or reverse primers (5′-GATTT GTTTTCTTTCCTGCC) to detect the nucleotide at position c.643 using the SNaPshot Multiplex kit (Applied Biosystems). Fragments were analyzed on the ABI-3730 sequencer (Applied Biosystems).

2.4. Western blot

Whole cell lysates were prepared and proteins separated on a 4–12% polyacrylamide gel (NuPage) and transferred to a Hybond ECL PVDF membrane. Nibrin was detected using the murine monoclonal antibody p95/NBS1-1D7 (GeneTex) and β-actin with a rabbit polyclonal antibody (Abcam). Several exposures of various lengths were made and the films scanned using the ScanMaker scanner (Mikrotek) and the images quantified using ImageQuant software (Molecular Dynamics).

2.5. Analysis of protein stabilization kinetics after proteasome or lysosome inhibition

SV40 transformed NBS and control fibroblasts were grown in medium supplemented with 10 μM MG-132 proteasome inhibitor (Boston Biochem) with or without 100 mM emetine (Sigma). After 0, 4, 8 and 12 h, cells were lysed, proteins extracted as detailed above and examined on a Western blot for p70-nibrin, p95-nibrin and β-actin as described. Densitometry was performed as described above and p70-nibrin or p95-nibrin signal intensities for each sample were normalized to the corresponding β-actin signal intensity. In LCLs, lysosomal hydrolases were inhibited by incubation of cells for 16 h in 50 μM chloroquine (Sigma). Nuclear and cytoplasmic proteins were isolated using the Proteo Extract Subcellular Proteome Extraction kit (Calbiochem) and examined by Western blot as detailed above. Rabbit polyclonal antibodies directed against β-tubulin (Abcam) and transcription factor E2F (Cell Signaling) were used to confirm cytoplasmic and nuclear extraction, respectively.

3. Results

3.1. p95-Nibrin and p70-nibrin in NBS patient cells

In the Western blot shown in Fig. 1, cell extracts from the cells used in this report were examined with antibodies directed against nibrin and β-actin. The two NBS cell lines, GM07166VA7 and NBS-1LBI, are SV40 immortalized fibroblasts homozygous for the c.657_661del5 founder mutation; they express only p70-nibrin, albeit at strikingly different levels. The control LCLs, 94P0197, express full length p95-nibrin, the cell line from a heterozygote for the c.657_661del5 founder mutation, 94P0111, shows lower expression of the full length protein, as expected. p70-Nibrin is not detected in heterozygous LCLs in Western blots but only after immunoprecipitation (Maser et al., 2001). 04P0014 LCLs show low levels of p95-nibrin carrying the p.Arg215Trp mutation. *NBN*-Arg^{215} and *NBN*-Trp^{215} transductants used in some experiments are also shown in Fig. 1.

3.2. p70-Nibrin turn-over in NBS patient cells

In the immortalized fibroblasts examined here (Fig. 1) there is an 8-fold difference in p70-nibrin expression, placing these two lines at the extremes of the previously reported spectrum in NBS patients (Kruger et al., 2007). As shown in Fig. 2A, specific inhibition of the proteasome with MG-132 leads to increasing p70-nibrin levels in both cell lines. Densitometry of Western blots allowed the quantitative analysis shown in Fig. 2B. Clearly, NBS-1LBI cells are much more responsive to proteasome inhibition than GM07166VA7 cells. In both cases, in the presence of the protein synthesis inhibitor emetine, p70-nibrin is not accumulated and its level remains stable,