Biochemical and Biophysical Research Communications 430 (2013) 72–77

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Establishment of a human cell line stably overexpressing mouse Nip45 and characterization of Nip45 subcellular localization

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ARTICLE INFO

Article history:
Received 2 November 2012
Available online 15 November 2012

Keywords:
Nip45
SUMO
Ubc9
Proteasome
PML bodies

ABSTRACT

The nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 interacting protein, Nfatc2ip (Nip45), has been implicated as a crucial coordinator of the immune response and of cellular differentiation in humans and mice, and contains SUMO-like domains in its C-terminal region. However, the significance of its N-terminal region and its correlation to the SUMO modification pathway remain largely uncharacterized. In this study, a human cultured cell line was established, in which FLAG-tagged mouse Nip45 (FLAG-mNip45) was stably overexpressed. Under standard, non-stressful conditions, we detected FLAG-mNip45 diffusely distributed in the nucleus. Intriguingly, proteasome inhibition by MG132 caused FLAG-mNip45, together with SUMOylated proteins, to localize in nuclear domains associated with promyelocytic leukemia protein. Finally, using an *in vitro* binding assay, we showed interaction of the N-terminal region of mNip45 with both free SUMO-3 and SUMO-3 chains, indicating that Nip45 may, in part, exert its function via interaction with SUMO/SUMOylated proteins. Taken together, our study provides novel information on a poorly characterized mammalian protein and suggests that our newly established cell line will be useful for elucidating the physiological role of Nip45.

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1. Introduction

Nip45 was originally identified as 'nuclear factor of activated T cells (NFAT) interacting protein of 45-kDa', a co-regulator with NFAT and the T helper 2 (Th2)-specific transcription factor, c-Maf, to induce IL-4 production [1]. It has also recently been shown to regulate members of the tumor necrosis factor (TNF)-receptor-associated factor (TRAF) family of proteins and has thus been implicated in the immune response [2–5] and cellular differentiation, such as receptor activator of NF-κB ligand (RANKL)-mediated osteoclast differentiation [6]. This protein can also recruit protein arginine *N*-methyltransferase 1 (PRMT1) to facilitate NFAT-driven transcriptional activity [7]. These data thus suggest that Nip45 is a multi-functional protein involved in multiple cellular signal transduction pathways, such as TNF–TRAF, RANKL–RANK, Toll and NF-κB pathways, indicating it as a potential therapeutic target for cancer and other human diseases. However, the molecular details of how Nip45 interacts with seemingly distinct signaling pathways remain largely unknown.

Amino acid sequence comparison identified Nip45 as a member of the Rad60-Esc2-Nip45 (RENi) protein family, which is conserved from yeast to humans [8]. RENi family members are distinguished from other protein families by their tripartite (bipartite for plant homologs) domain architecture. An N-terminal polar/charged low-complexity segment and two consecutive unique small ubiquitin-related modifier (SUMO)-like domains, referred to as SLDs, in the C-terminal region can be detected by sequence-based domain and fold-recognition methods as well as by a structure-based similarity method [8]. For instance, mouse and human Nip45 proteins (mNip45 and hNip45) consist of 421 and 419 amino acid residues, respectively, and show 71% identity (79% similarity) with each other over their entire polypeptide sequences. Both contain two SLDs in their C-terminal regions: the N-terminal and C-terminal domains of the C-terminal region are designated as SLD1 and SLD2, respectively (see Fig. 1A). Polypeptide sequence comparison of mNip45-SLD1 versus hNip45-SLD1 shows 90% identity (96% similarity), and a comparison of their SLD2s shows 92% identity (95% similarity). The N-terminal regions of both mNip45 and hNip45 contain an excess of polar/charged residues and have only 60% identity (68% similarity) with a low complexity, implying that these regions are most likely distinct from the C-terminal SLDs and are conformationally flexible segments without inherent structural preference [9,10].

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http://dx.doi.org/10.1016/j.bbrc.2012.11.020

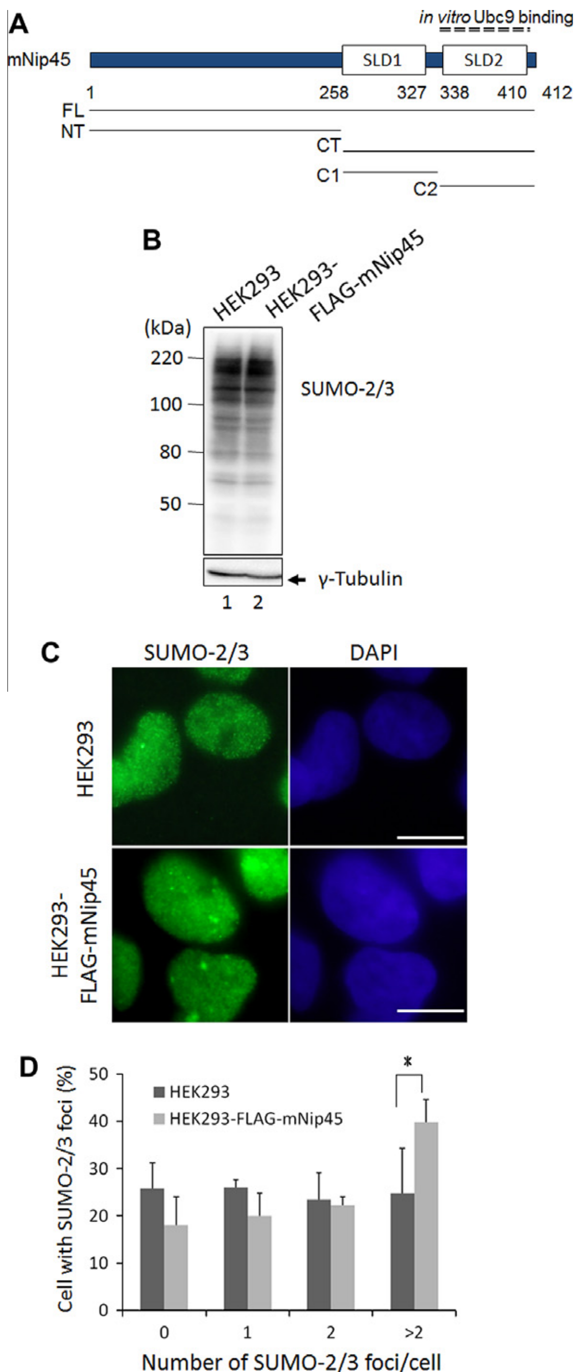

Fig. 1. Detection of SUMO/SUMOylated proteins in FLAG-mNip45 expressing cells. (A) Schematic representation of mNip45. The numbers correspond to the amino acid residues in mNip45. The positions of the SUMO-like domains (SLD1 and SLD2) are shown [11]. The full length (FL), N-terminal (NT), C-terminal (CT), SLD1-containing (C1), and SLD2-containing (C2) domain constructs used in this study are represented as thin black lines. (B) Non-transfected HEK293 cells (−: lane 1) and FLAG-mNip45 expressing cells (+: lane 2) were cultured under standard conditions. Cell lysates were dissolved in SDS-sample buffer, followed by immunoblot analysis using anti-SUMO-2/3 antibody. To confirm whether cellular proteins were equally blotted on each membrane, the membranes were re-probed with anti-tubulin antibody (lower panel). The positions of protein size markers are shown at left. (C) Non-transfected HEK293 cells (upper panel) and FLAG-mNip45 cells (lower panel) were cultured under standard conditions and subjected to indirect-immunofluorescence assays using anti-FLAG (left) and SUMO-2/3 (center) antibodies. DAPI staining was used for nuclear staining (right panel). Bar indicates 20 μm. (D) The SUMO-2/3 nuclear foci were quantified; cell numbers shown are the mean ± standard deviation (SD) of three independent experiments ($^*p < 0.05$ by Student's *t*-test). White bars represent the values of non-transfected cells; black bars, FLAG-mNip45 expressing cells.

Sekiyama et al. have reported the crystal structures of mNip45-SLD2, both in free form and complexed with an E2 SUMO-conjugating enzyme, Ubc9. They showed that mNip45-SLD2 binds to Ubc9 in a manner almost identical to that of SUMO paralogs, SUMO-1 and SUMO-2/3 (see Fig. 1A) [11]. It has also been reported that the SLDs of Rad60, a Nip45 homolog in the fission yeast *Schizosaccharomyces pombe*, interacts with Ubc9 [12]. Comparison of structural data between mNip45-SLD2:Ubc9 and Rad60-SLD2:Ubc9 complexes suggests that the interaction between RENi family members and Ubc9 appears to be evolutionarily conserved [11–13], arguing that RENi proteins act as SUMO-stable fusion proteins mimicking SUMO. Although it has been shown that mNip45-SLD2 inhibits *in vitro* elongation of SUMO chains, whether Nip45 confers such an inhibitory effect on the polymerization of SUMOs *in vivo* remains unclear. In addition, in contrast to the C-terminal region of Nip45, the N-terminal region is poorly characterized.

In this study, we established a human cell line, in which FLAG-tagged mouse Nip45 (FLAG-mNip45) was stably overexpressed. Using this cell line, the involvement of Nip45 with the metabolism of SUMO/SUMOylated proteins in response to proteasome inhibition was revealed. We also found that the N-terminal region of Nip45 has the potential to interact with SUMO-3 and SUMO-2/3 chains, which explains one *in vivo* action of Nip45. Our study provides novel information on a poorly characterized mammalian protein and suggests that our newly established cell line will be useful for elucidating the physiological role of Nip45.

2. Materials and methods

2.1. Plasmids

To generate Nip45 expression vectors, mNip45 cDNA was amplified by polymerase chain reaction (PCR) using appropriate primers (primer sequences available upon request). The generated PCR fragments were cloned into pET28 (Novagen), pGEX 4T-1(GE Healthcare), and pcDNA3 (Invitrogen). The deletion mutants of mNip45 were also generated by PCR. SUMO-1/2/3 expression plasmids were used as described previously [14,15].

2.2. Antibodies

Two rat monoclonal antibodies, against SUMO-2/3 and against SUMO-1, were described previously [14]. The other antibodies used in this study were anti-SUMO-1, anti-SUMO-2/3, anti-Ubc9 (Cell signaling TECHNOLOGY and BD), anti-Nip45, anti-His_6, anti-GST, anti-γ-tubulin, anti-PML (Santa Cruz Biotechnology), anti-K48/K63-specific ubiquitin (Millipore), and anti-FLAG M2 (Sigma–Aldrich).

2.3. Recombinant protein expression and GST-pull-down assay

Expression of recombinant proteins and purifications of GST fusion proteins and His_6 fusion proteins were carried out as previously described [14]. Standard conditions were as follows: bacterially expressed GST and GST fusion proteins were immobilized on glutathione-Sepharose beads (GE healthcare), and were incubated with lysate prepared from either *Escherichia coli* in phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Proteins associated with the beads were analyzed by sodium dodecyl sulfate (SDS)–PAGE, followed by immunoblot analysis [14,15]. Polyubiquitin chains (ubi_{1-7}-K48- and -K63-linked) were purchased from Boston Biochem.

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