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Biochemical and Biophysical Research Communications 337 (2005) 116–120
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Interaction of NUB1 with the proteasome subunit S5a

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Received 18 August 2005
Available online 13 September 2005

Abstract

NUB1 interacts with a ubiquitin-like protein NEDD8 to target the NEDD8 monomer and neddylated proteins to the proteasome for degradation. Therefore, NUB1 is thought to be a potent downregulator of NEDD8 conjugation system. Since NUB1 possesses a UBL domain, which was previously shown to be an S5a-interacting motif in RAD23/HHR23, we initially hypothesized that NUB1 interacts with the S5a subunit of the proteasome through its UBL domain. To examine this, we performed an in vitro GST pull-down assay and a yeast two-hybrid assay. Unexpectedly, our studies revealed that NUB1 directly interacts with the S5a subunit through its C-terminal region between amino acid residues 536 and 584, not through its UBL domain. Although the UBL domain was not an S5a-interacting motif in NUB1, our further studies revealed that the UBL domain is required for the function of NUB1.

Keywords: NUB1; NUB1L; AIPL1; NEDD8; Ubiquitin; Proteasome; S5a; UBL; UBA

NEDD8 is an 81-amino acid protein that shares 60% identity and 80% homology with ubiquitin. NEDD8 conjugates to target proteins, and this conjugation (neddylation) is catalyzed by enzymatic reactions, in a manner analogous to ubiquitination [1,2]. So far, NEDD8 has been reported to conjugate to a limited number of target proteins, including cullin family members, the von Hippel–Lindau tumor suppressor gene product (pVHL), and p53 [3–5]. Because neddylation modifies the function of these target proteins, the neddylation system seems to regulate many important biological events [2,4,5].

Recently, we identified a novel downregulator of the neddylation system, NUB1 [6], which is a NEDD8-interacting protein composed of 601 amino acid residues with a calculated molecular mass of 69.1 kDa. It possesses a ubiquitin-like (UBL) domain at the N-terminal region and two ubiquitin-associated (UBA) domains at the C-terminal region. Further, it is an interferon-inducible protein and predominantly localizes in the nucleus. In a biochemical analysis, we found that NUB1 recruits NEDD8 and its conjugates to the proteasome for degradation, making NUB1 a downregulator in the neddylation system [7].

Because some UBL domains have been shown to interact with the S5a subunit of the 26S proteasome [8], we hypothesized that NUB1 interacts with the S5a subunit through its UBL domain to target NEDD8 to the proteasome. To test this hypothesis, we performed a GST pull-down assay and a yeast two-hybrid assay. In addition, we examined whether the UBL domain is essential for NUB1 function.

Materials and methods

Cell culture. COS-M6 cells were generous gifts of Dr. Steven Goldring of Harvard Medical School. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies. Mouse anti-RH antibody specific to the amino acid sequences RGSHHHH and GGSHHHH was purchased from Qiagen (Santa Clara, CA). GST-12, a mouse anti-glutathione-*S*-transferase (GST) antibody, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-FLAG antibody (M5) and rabbit polyclonal anti-actin antibody (specific for the C-terminal actin fragment) were purchased from Sigma (St. Louis, MO).

Plasmid construction. To express RH-tagged S5a and GST-fusion proteins in *Escherichia coli* BL21 cells, pTrcHis plasmid (Invitrogen) and

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doi:10.1016/j.bbrc.2005.09.014

pGEX-2TK plasmid (Amersham Pharmacia Biotech) were used, respectively. The cDNA inserts in these plasmids, including human S5a [7], wild-type NUB1 [6], and HHR23B [9], were prepared from other plasmids constructed previously in our laboratory. The cDNAs of truncated NUB1 were amplified by a polymerase chain reaction (PCR) using appropriate primers and a cDNA of wild-type NUB1.

To express proteins tagged with epitope at the N-terminus in COS-M6 cells, pcDNA3/RH-N and pcDNA3/FLAG-N were used as described previously [7,10]. The human cDNAs in these plasmids were also described previously. These include NEDD8 [1], the glucocorticoid receptor (GR) [11], and NUB1 [6]. The cDNA of truncated NUB1 (amino acid residues 148–601) lacking a UBL domain was amplified by PCR. These cDNAs were inserted into the aforementioned plasmid vectors, and the plasmids were transfected into COS-M6 cells using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN). The transfected cells were then harvested for Western blotting 20 h after transfection.

GST pull-down assay. RH-tagged S5a and GST-fusion proteins were expressed in *E. coli* BL21 cells, as described previously [12], using pTrcHis plasmid (Invitrogen) and pGEX-2TK plasmid (Amersham Pharmacia Biotech), respectively. Cells were then resuspended in lysis buffer (25 mM Tris–HCl [pH 7.5], 100 mM NaCl, and 0.1% NP-40) containing protease inhibitor cocktail (Roche) and then lysed by brief sonication. The GST-fusion proteins were purified as described previously [12]. The crude bacterial lysate containing RH-S5a was centrifuged at 14,000*g* for 5 min, and the supernatant was incubated for 3 h at room temperature with GST-fusion proteins immobilized on glutathione–Sepharose beads (Amersham Biosciences). Next, the beads were washed four times with lysis buffer. The precipitated proteins on the beads were solubilized in sample treating buffer containing 2% SDS and 5% β-mercaptoethanol. The solubilized proteins were analyzed by Western blot analysis using anti-RH antibody.

Yeast two-hybrid analysis. The cDNAs used in the yeast two-hybrid assay had been previously prepared. These included S5a, wild-type NUB1, full-length NUB1 with L573A and L577A substitutes, truncated NUB1, truncated NUB1 with L573A and L577A substitutes, wild-type NUB1L, and HHR23B [9,13]. To examine the in vivo interaction of S5a with wild-type NUB1, mutant NUB1, wild-type NUB1L, or HHR23B, the yeast MATCHMAKER two-hybrid system 3 (Clontech) was used as described previously [14]. The cDNA of human S5a was subcloned into pGADT7 (GAL4 DNA-activating domain vector for Gal4-AD fusion), while the other cDNAs were subcloned into pGBKT7 (Gal4 DNA-binding domain vector for Gal4-BD fusion). The plasmids for the two fusion constructs were cotransfected into AH109 yeast cells, and then the yeast two-hybrid interaction was tested as described previously [9].

Western blotting. Protein samples were treated at 45 °C for 1 h in 2% SDS treating solution containing 5% β-mercaptoethanol. After SDS–PAGE, Western blotting was performed using the protocol provided with the ECL detection system (Amersham Biosciences). As a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (Santa Cruz Biotechnology) was used.

Results

In vitro interaction of S5a with truncated NUB1

At the N-terminal region, NUB1 possesses a UBL domain, which is thought to be an S5a-binding motif for the interaction with the 26S proteasome [8]. Therefore, we initially hypothesized that NUB1 binds to the 26S proteasome through the interaction between the UBL domain of NUB1 and the S5a subunit of the proteasome. Indeed, in our previous study, we demonstrated the physical interaction between NUB1 and the S5a subunit of the proteasome [7]. However, we did not define whether the UBL domain of NUB1 interacts with the S5a subunit. To determine this, we performed a GST pull-down assay (Fig. 1). In

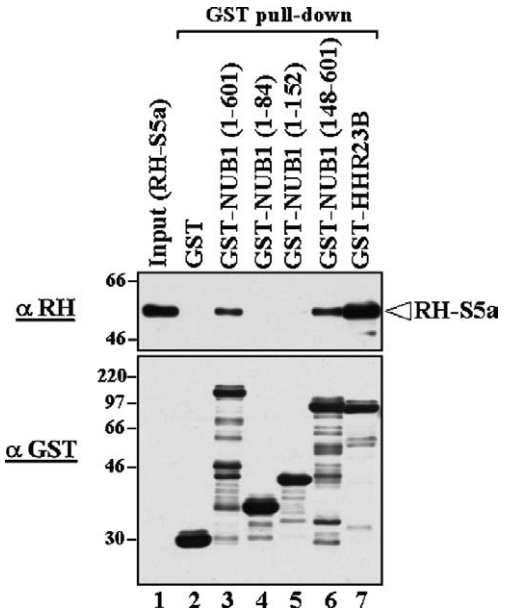

Fig. 1. GST pull-down assay done to examine the in vitro interaction between S5a and NUB1 mutants. RH-tagged S5a was expressed in bacteria. The bacterial lysate containing RH-S5a was precipitated by GST (lane 2), GST-NUB1 (wild type) (lane 3), GST-NUB1 mutant with a C-terminal deletion (lanes 4 and 5) or an N-terminal deletion (lane 6), or GST-HHR23 (lane 7). The precipitates were analyzed by Western blotting using anti-RH antibody to detect RH-S5a (upper panel) and anti-GST antibody to detect GST, GST-NUB1, or GST-HHR23 (lower panel).

bacteria, we expressed GST alone and GST-fused full-length NUB1 and its truncated mutants, including $NUB1^{1–84}$, $NUB1^{1–152}$, and $NUB1^{148–601}$. $NUB1^{1–84}$ is an N-terminal fragment of NUB1 and does not possess either UBL or UBA domains. $NUB1^{1–152}$ is a longer N-terminal fragment and possesses a UBL domain but not UBA domains. $NUB1^{148–601}$ is a C-terminal fragment and possesses two UBA domains but not a UBL domain (see Fig. 2D). As a positive control, we used HHR23B, which is known as an S5a-interacting protein [15]. These GST-fusion proteins were purified with glutathione beads, and then RH-tagged S5a was precipitated using these protein-coated beads. The precipitates were analyzed by Western blotting using anti-RH antibody or anti-GST antibody. As shown in Fig. 1, RH-S5a could not be precipitated by GST, GST-$NUB1^{1–84}$, or GST-$NUB1^{1–152}$ (lanes 2, 4, and 5), whereas it could be precipitated by GST-$NUB1^{1–601}$ (full length) or GST-$NUB1^{148–601}$, as well as by GST-HHR23B (lanes 3, 6, and 7). These results indicated that NUB1 directly interacts with the S5a subunit through its C-terminal region between amino acid residues 148 and 601 but not through its UBL domain.

Identification of S5a-binding site on NUB1

Since we found that S5a directly binds to the C-terminal region of NUB1 (amino acid residues 148–601) in the in vitro interaction assay, we next attempted to precisely identify the S5a-binding site on NUB1. First, we generated cDNAs of two truncated NUB1s, N-terminal half (amino