Biochemical and Biophysical Research Communications 428 (2012) 438–444

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The N-terminal ectodomain of Ninjurin1 liberated by MMP9 has chemotactic activity

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ARTICLE INFO

Article history:
Received 3 October 2012
Available online 7 November 2012

Keywords:
Ninjurin1
Posttranslational modification
MMP9
Proteolytic cleavage
Chemotaxis

ABSTRACT

Ninjurin1 is known as an adhesion molecule promoting leukocyte trafficking under inflammatory conditions. However, the posttranslational modifications of Ninjurin1 are poorly understood. Herein, we defined the proteolytic cleavage of Ninjurin1 and its functions. HEK293T cells overexpressing the C- or N-terminus tagging mouse Ninjurin1 plasmid produced additional cleaved forms of Ninjurin1 in the lysates or conditioned media (CM). Two custom-made anti-Ninjurin1 antibodies, Ab_{1-15} or $Ab_{139-152}$, specific to the N- or C-terminal regions of Ninjurin1 revealed the presence of its shedding fragments in the mouse liver and kidney lysates. Furthermore, Matrix Metalloproteinase (MMP) 9 was responsible for Ninjurin1 cleavage between Leu^{56} and Leu^{57}. Interestingly, the soluble N-terminal Ninjurin1 fragment has structural similarity with well-known chemokines. Indeed, the CM from HEK293T cells overexpressing the GFP-mNinj1 plasmid was able to attract Raw264.7 cells in trans-well assay. Collectively, we suggest that the N-terminal ectodomain of mouse Ninjurin1, which may act as a chemoattractant, is cleaved by MMP9.

1. Introduction

Ninjurin1 was originally identified by Araki et al. as a small-size adhesion molecule with 152 amino acids (aa, ~17 kDa) consisting of an N-terminal (1–71 aa) and C-terminal (139–152 aa) ectodomain, two transmembrane domains (72–100 aa and 111–138 aa), and an intercellular region (101–110 aa) [1]. In particular, the 12 residues on the N-terminal ectodomain of Ninjurin1, from Ala^{26} to Val^{37}, is crucial for its homophilic binding activity [2], promoting neurite extension and axonal regeneration after sciatic nerve injury following transection [1]. Recently, we demonstrated that Ninjurin1 is selectively upregulated in myeloid cells and inflamed endothelial cells to mediate leukocyte trafficking in rat experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis [3]. Furthermore, Ninjurin1 blockage using a synthetic peptide specific to its homophilic binding domain alleviates EAE susceptibility [4]. Recently, Ninjurin1 was found to be involved in the crawling of T cells on the intraluminal surface of CNS vessels in rat EAE [5]. These results demonstrate that the homophilic binding activity on the N-terminal ectodomain of Ninjurin1 enhances leukocyte trafficking by promoting the leukocyte-endothelium adhesions.

Some adhesion molecules involved in leukocyte trafficking alter their binding property by proteolytic cleavage such as CD44 [6], L-selectin [7], and Fractalkine [8]. In *Drosophila*, the N-terminal ectodomain of Ninjurin A, having 98% homology with that of mouse Ninjurin1, can be cleaved by MMP and triggers loss of cell adhesion [9].

Therefore, we investigated whether proteolytic cleavage occurs in mouse Ninjurin1 and what the functions are of those cleaved fragments. We found the shedding fragments of mouse Ninjurin1 using N- or C-terminus tagging plasmids *in vitro* as well as N- or C-terminal specific anti-Ninjurin1 antibodies *in vivo*. Through li-

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http://dx.doi.org/10.1016/j.bbrc.2012.10.099

quid chromatography–mass spectrometry (LC–MS) analysis, the cleavage site was defined as the residue between Leu^{56} and Leu^{57} and this cleavage likely requires MMP9 action, but not MMP2. Furthermore, the liberated N-terminal ectodomain of Ninjurin1 which has a similar structure with chemokines showed the chemotactic activity for Raw264.7 cells. Altogether, we propose that mouse Ninjurin1 is cleaved by MMP9 and its liberated fragments may act as a novel chemoattractant for modulating inflammatory responses.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from Orient Bio. Inc. and maintained under pathogen-free condition in the animal housing facilities of the College of Pharmacy at the Seoul National University for the period of experiments by the Committee for Care and Use of Laboratory Animals at the Seoul National University (SNU-101011-1).

2.2. Cell culture and transfection

HEK293T and Raw264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and maintained in an incubator with a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C. All transfection in HEK293T cells were implemented using polyethylenimine (PEI) reagent.

2.3. Antibodies and vector constructs

For custom-made rabbit anti-mouse Ninjurin1 antibodies, a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide bearing mouse Ninjurin1 residues 1–15 (Ab_{1-15}) or 139–152 ($Ab_{139-152}$) was used to immunize rabbits following standard procedures (Abfrontier Inc., Korea), and both anti-Ninjurin1 antibodies were purified each with antigen-specific affinity chromatography. The Ab_{1-15} or $Ab_{139-152}$ was used for Western blot analysis. A normal rabbit IgG (purified by Protein A column, Upstate) or Ab_{1-15} was used for neutralization of the liberated GFP-mNinj1 in the trans-well assay *in vitro*.

Several mouse Ninjurin1 (NM_013610) expression vectors were constructed as described previously [10]. The pCMV-Tag2B or $pCS2^+$-GFP was used as a backbone for the construction of N-terminus tagging vectors, Flag-mNinj1 or GFP-mNinj1, respectively. The 3×Flag tagging mouse Ninjurin1 (3×Flag-mNinj1) vector was made with the pCMV14 backbone for the C-terminus tagging system. Using the $pCS2^+$ GFP backbone, some truncated vectors including GFP-$mNinj1_{1-71}$ (C-terminal region deletion) or GFP-$mNinj1_{72-152}$ (N-terminal region deletion) were constructed. The non-tagging mouse Ninjurin1 vector ($pcDNA3.1^+$ myc/his) was designed by adding a stop codon at the end of the Ninjurin1 sequence.

2.4. Immunoprecipitation and western blotting

Tissues or cells were lysed in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1X a protease inhibitor cocktail solution (Calbiochem). The conditioned media (CM) or lysates were immunoprecipitated in buffer solution containing 20 mM Tris–HCl (pH 7.4), and 150 mM NaCl, and immunoblotted with the corresponding primary antibodies such as Ninjurin1 (Ab_{1-15} and $Ab_{139-152}$), GFP (Abcam), Flag (Sigma), or myc antibody (Santa Cruz).

2.5. In vitro MMPs cleavage assay

Recombinant MMP2 (R&D), MMP9 (R&D), and the catalytic domain of MMP9 (Cat-MMP9, Peprotech) were commercially purchased. Non-tagging mouse Ninjurin1 protein was immunoprecipitated with the Ab_{1-15} antibody. After 1 mM p-aminophenylmercuric acetate (APMA) activation, the concentration of MMP2 or MMP9 was determined. The APMA-activated MMPs were incubated with the immunoprecipitated mouse Ninjurin1 protein with or without GM6001 (10 μM), a pan-MMP inhibitor in a buffer solution containing 0.2 M NaCl, 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.5), 0.005% Brij, and 0.0025% NaN_3 for 16 h at 37 °C. Reaction products were analyzed by Tris–glycine SDS–PAGE and western blot with the $Ab_{139-152}$ antibody.

To determine the cleavage sites of Ninjurin1, three kinds of peptides, PEP_{1-30}, PEP_{21-50}, and PEP_{41-70}, were chemically synthesized using solid phase Fmoc chemistry on a PeptrEx synthesizer (Peptron Inc.) and purified by liquid chromatography (LC) (>90% purity). All peptides were verified by mass spectrometry (MS) analysis. Each peptide was incubated with recombinant MMP9 at an enzyme:peptide ratio of 1:10 (w/w) for 16 h at 37 °C in a buffer solution containing 0.2 M NaCl, 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.5), 0.005% Brij, and 0.0025% NaN_3. Reaction products were analyzed by LC–MS.

2.6. Transmigration assay

For the migration assay, Raw264.7 cells labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5 min were added to the upper chamber of the trans-well (6.5 mm diameter, 8 μm pore size, Costar). The GFP or GFP-mNinj1 overexpressed CM was applied into the lower chamber to examine the chemotactic activity. After incubation for 8 h, the trans-well was fixed with 4% PFA and its upper side was cleaned with cotton and mounted. Pictures were taken at six positions using microscopy (Axiovert M200, Carl Zeiss) and the migrated cells were analyzed by determining the percentage of CSFE-labeled cells.

2.7. Data analysis and statistics

All data are presented as the means ± s.e.m and expressed as relative percentages and fundamental units. Statistical significance was calculated using ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The N-terminal ectodomain of the overexpressed Ninjurin1 is cleaved in vitro

To investigate whether mammalian Ninjurin1 can be cleaved, we constructed a couple of mouse Ninjurin1 plasmids tagged with Flag (Flag-mNinj1) at the N-terminus and with 3×Flag (3×Flag-mNinj1) at the C-terminus (Fig. 1A). HEK293T cells overexpressed with Flag-mNinj1 produced one major band corresponding to the expected molecular weight (~23 kDa, black arrow) (Fig. 1B), while 3×Flag-mNinj1 transfectants contained three additional bands (~15/16/19 kDa, red arrowheads) with molecular weights below the full-length (~21 kDa, black arrow) protein that were regarded as its cleaved fragments (Fig. 1C). Next, we examined whether the cleaved fragments were secreted into the conditioned media (CM). After overexpressing mouse Ninjurin1 plasmid tagged with GFP at