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

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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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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

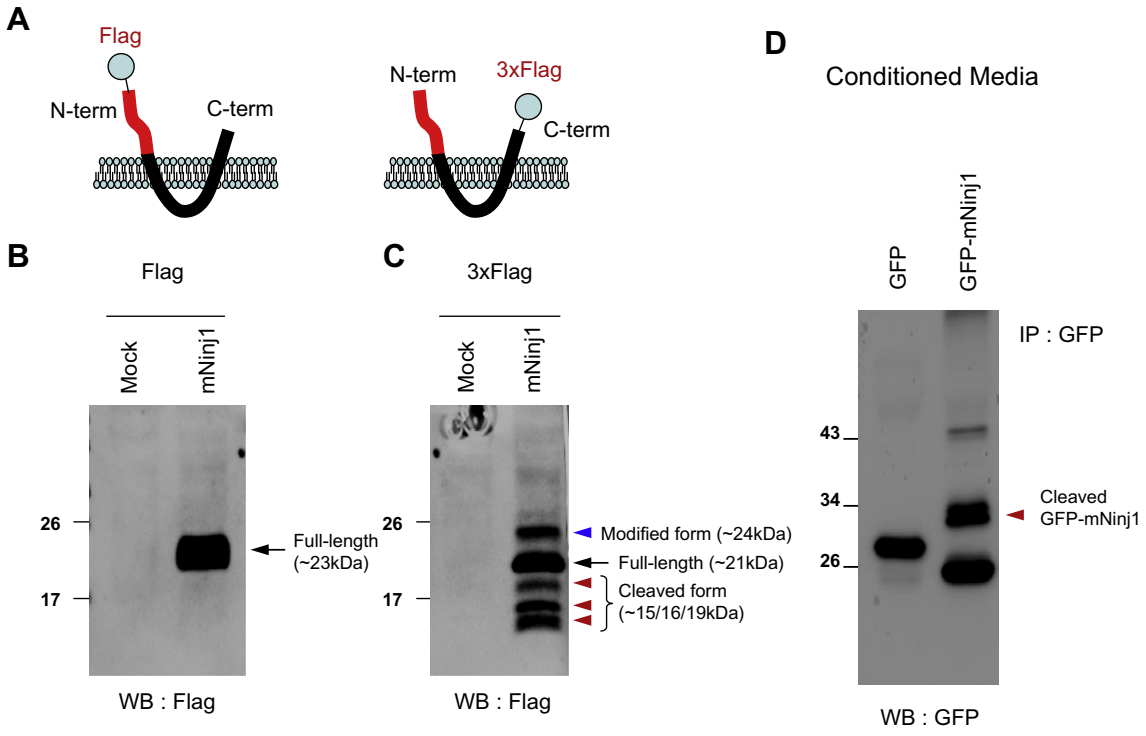

Fig. 1. The cleavage of the N-terminal ectodomain of mouse Ninjurin1 *in vitro*. (A) Schematic diagram of the two N- or C-terminus tagging mouse Ninjurin1 plasmid, Flag-mNinj1 or 3×Flag-mNinj1, respectively. (B and C) Flag-mNinj1, 3×Flag-mNinj1 or their corresponding mock plasmids were overexpressed in HEK293T cells. After 24 h, cells were harvested and Western blotting was performed with anti-Flag antibody. (B) Black arrow (~23 kDa) depicts the full-length Flag-mNinj1. (C) Black arrow (~21 kDa) indicates the full-length 3×Flag-mNinj1 and the red arrowheads indicate the expected cleavage fragments or its modified forms (~15/16/19 kDa). The blue arrowhead (~24 kDa) indicates presumptive modified form of the full-length 3×Flag-mNinj1. (D) GFP or GFP-mNinj1 was transfected in HEK293T cells, and their CM were collected and immunoprecipitated with anti-GFP antibody. Western blotting was performed with anti-GFP antibody. The red arrowhead depicts the cleaved GFP-mNinj1 fragments in CM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the N-terminus (GFP-mNinj1) in HEK293T cells, CM was immunoprecipitated with anti-GFP antibody. Western blotting with anti-GFP antibody clearly showed the GFP-bearing fragments of Ninjurin1 in CM (Fig. 1D). Collectively, these results indicate that Ninjurin1 can be cleaved at the N-terminal ectodomain and secreted into the CM *in vitro*.

3.2. The shedding fragments of Ninjurin1 are found in the mouse liver and kidney lysates

Next, we asked whether the cleavage of Ninjurin1 occurs *in vivo*. To detect endogenous Ninjurin1, we made a couple of anti-mouse Ninjurin1 antibodies, Ab_{1-15} and $Ab_{139-152}$ which selectively recognize N- or C-terminal region of Ninjurin1, respectively. The specificity of each antibody was evaluated using two truncated GFP-mNinj1 plasmids, GFP-mNinj1$_{72-152}$ or GFP-mNinj1$_{1-71}$, which lacks of its N- or C-terminal region, respectively. As expected, the Ab_{1-15} antibody recognized well the full-length and GFP-mNinj1$_{1-71,}$ but not the GFP-mNinj1$_{72-152}$ (Fig. 2A, middle), whereas the $Ab_{139-152}$ antibody detected both the full-length and GFP-mNinj1$_{72-152}$, but not the GFP-mNinj1$_{1-71}$ (Fig. 2A, right), indicating that the specificities of these antibodies are enough to distinguish cleaved Ninjurin1 fragments.

Using these antibodies, we performed Western blot analysis with lysates from several organs in the C57BL/6 mouse. Both antibodies recognized the endogenous full-length Ninjurin1 (~17 kDa, black arrow) with similar patterns, whereas only $Ab_{139-152}$, not Ab_{1-15}, antibody was capable of detecting one or more additional bands (~11/12/15 kDa, red arrowheads) below full-length Ninjurin1 in the mouse liver and kidney lysates (Fig. 2B). Moreover, Western blotting with the $Ab_{139-152}$ antibody after immunoprecipitation of mouse liver lysates with each antibody, IgG isotype, Ab_{1-15}, and $Ab_{139-152}$ antibody, clarified that Ninjurin1 is fragmented at the N-terminal ectodomain region (Fig. 2C). Collectively, consistent with the *in vitro* observations, the fragmentations of the Ninjurin1 N-terminal ectodomain are found in the mouse liver and kidneys *in vivo*.

3.3. MMP9 contributes to the cleavages of Ninjurin1 in between Leu[56] and Leu[57]

To elucidate which proteases might be responsible for the cleavage of Ninjurin1, we first screened putative proteases using various bioinformatics software such as Sitepredition [11] and MEROPS [12]. These softwares provided us with MMP2 and MMP9 as possible candidates (data not shown), and which is consistent with the report that MMP cleaves the N-terminal ectodomain of Ninjurin1 A in *Drosophila* [9]. Therefore, we speculated that MMP2 and/or MMP9 are able to shed mouse Ninjurin1. To prove this hypothesis, an *in vitro* MMP cleavage assay was performed as described in the Section 2. MMP9 incubated with 4-aminophenylmercuric acetate (APMA), a chemical activator of MMPs, cleaved the full-length Ninjurin1 protein (~17 kDa, black arrow) into a smaller protein (~11 kDa, red arrowhead), while MMP2 did not (Fig. 3A). Furthermore, truncated MMP9 with catalytic domain, Cat-MMP9, was able to cleave the Ninjurin1 protein, which was inhibited by treatment with GM6001, a pan-MMP inhibitor (Fig. 3B), clearly demonstrating the role of MMP9 in the fragmentation of Ninjurin1.

Next, to identify the cleavage sites on Ninjurin1 mediated by MMP9, we synthesized three kinds of peptides, PEP$_{1-30}$, PEP$_{21-50}$, and PEP$_{41-70}$, corresponding to the indicated region of the N-terminal ectodomain of mouse Ninjurin1 (mNinj1$_{1-70}$). After each

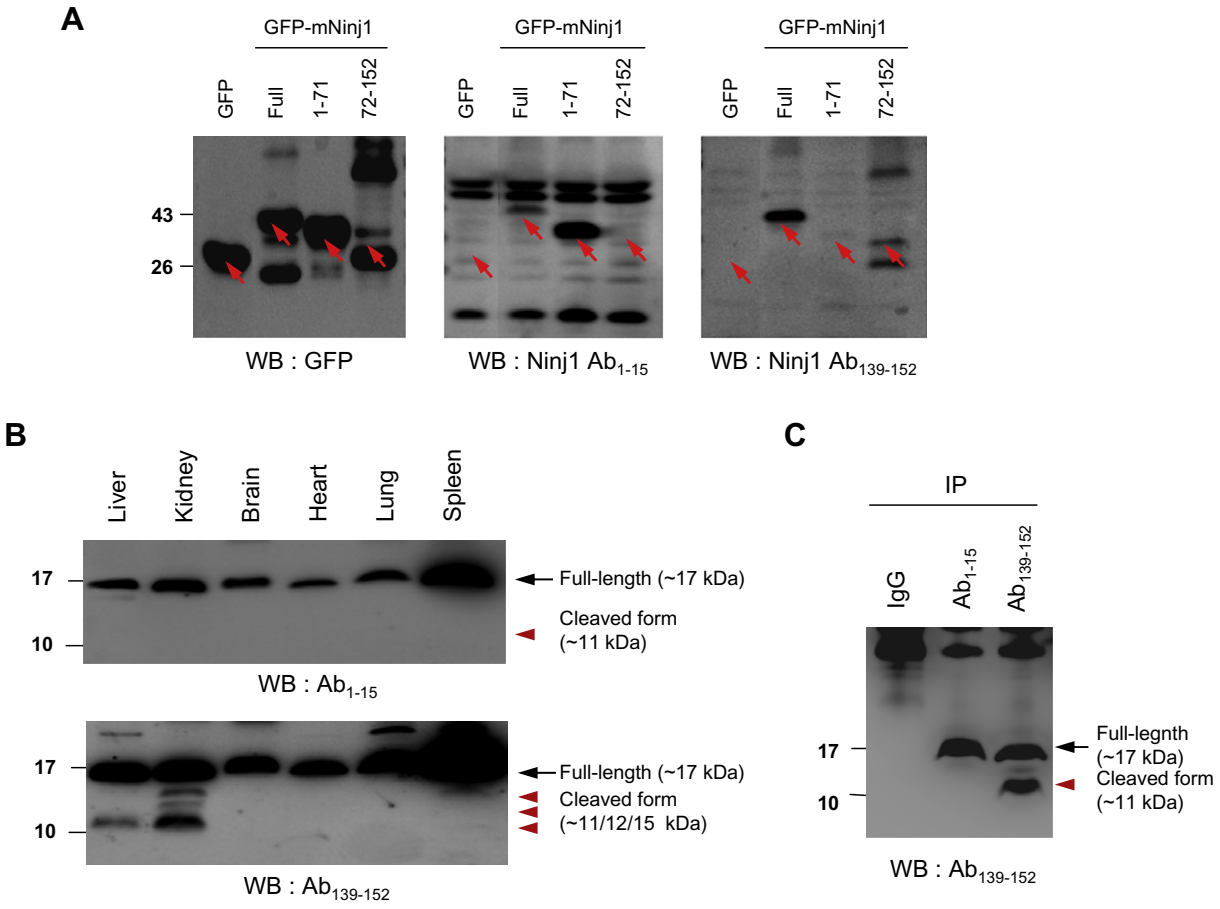

Fig. 2. The cleavage of Ninjurin1 in the mouse liver and kidneys lysates. (A) The construction of two custom-made rabbit anti-mouse Ninjurin1 antibodies which are specific to the N- or C-terminal region of mouse Ninjurin1, Ab_{1-15} or $Ab_{139-152}$, respectively. Antibodies were purified with peptide-bearing column for each respective antigen. A couple of plasmids truncated with the C- or N-terminal domain, GFP-mNinj1_{1-71} or GFP-mNinj1_{72-152}, were constructed and overexpressed in HEK293T cells. After 24 h, the lysate was blotted with anti-GFP (left) or anti-Ninjurin1 antibody, Ab_{1-15} (middle) and $Ab_{139-152}$ (right). Red arrows indicate the product corresponding to the expected product of each plasmid. (B) Several tissues were isolated from C57BL/6 mice and Western blot analysis was performed with Ab_{1-15} or $Ab_{139-152}$. The black arrow indicates the endogenous mouse Ninjurin1 (~17 kDa) and the red arrowheads indicate bands shows its cleaved or modified forms (~11/12/15 kDa). (C) The liver lysates of mice were immunoprecipitated with normal rabbit IgG, Ab_{1-15}, or $Ab_{139-152}$ antibodies, and Western blot analysis was conducted with the $Ab_{139-152}$ antibody. The black arrow indicates the full-length mouse Ninjurin1 (~17 kDa) and the red arrowhead shows its cleaved form (~11 kDa). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

peptide was incubated with the recombinant Cat-MMP9, the cleaved residues were determined by LC-MS/MS analysis with Thermo Finnigan's LCQ Deca ion trap mass spectrometer. Neither PEP_{1-30} nor PEP_{21-50} was fragmented by MMP9 (data not shown). However, three additional peaks, ①, ②, and ③, were found in the ion chromatogram graph of PEP_{41-70} (Fig. 3C). MS/MS spectra obtained in the LC-MS/MS analysis were searched against Ninjurin1 amino acid sequence by SEQUEST algorithm, which revealed each peak was matched with a cleaved fragment as follows: ② matched to PEP_{57-70} (LMANASQLKAVVEQ), ① matched to oxidized form of PEP_{57-70} at Methionine residue (ox-Met containing PEP_{57-70}), and ③ matched to PEP_{41-56}, YANKKSAAESMLDIAL) (data not shown). The LC-MS/MS analysis results clearly demonstrated that the site for MMP9-mediated Ninjurin1 cleavage is in between Leu^{56} and Leu^{57} on its N-terminal ectodomain.

Based on our observation, there are likely at least two or more cleavage sites in the 3×Flag-mNinj1 protein *in vitro* (Fig. 1C, red arrowheads) and in the endogenous Ninjurin1 in the mouse kidney lysates (Fig. 2B, red arrowheads), respectively. To determine which band corresponds with the cleavage event between Leu^{56} and Leu^{57}, an additional 3×Flag-mNinj1_{57-152} plasmid was designed to have the remaining portion of Ninjurin1 after cleavage and was compared to the full-length 3×Flag-mNinj1 plasmid. The overexpression of 3×Flag-mNinj1_{57-152} in HEK293T cells generated a band corresponding to the lowest one (red arrowhead, ~15 kDa) among the products of the full-length 3×Flag-mNinj1 (Fig. 3D). Altogether, these results clearly suggest that MMP9 is responsible for the cleavage of Ninjurin1 between Leu^{56} and Leu^{57}.

3.4. The liberated N-terminal fragment of Ninjurin1 has chemotactic properties

To explore the biologic functions of the cleaved fragment of Ninjurin1 (mNinj1_{1-56}), its structural properties were estimated using computational methods: Garnier–Osguthorpe–Robson (GOR) methods for secondary structure prediction [13] and DisEMBL database for the disordered region prediction [14]. The mNinj1_{1-56} contains the disordered N-terminal amino acids (1–11 aa), a β-sheet (15–39 aa), and a N- or C-terminal α-helix (1–10 aa and 40–56 aa) (Fig. 4A). Intriguingly, several chemokines share a highly conserved tertiary structure consisting of a disordered N-terminal domain, a three-stranded anti-parallel β-sheet, and a C-terminal α-helix despite their low sequence similarity [15]. Indeed, after the multiple alignments of mNinj1_{1-56} with well-known chemokines including SDF-1α [16], fractalkine [17], and MIP-1β [18] using T-coffee software, a consistency-based multiple sequence alignment program [19], the comparison of their secondary structures revealed a close structural similarity

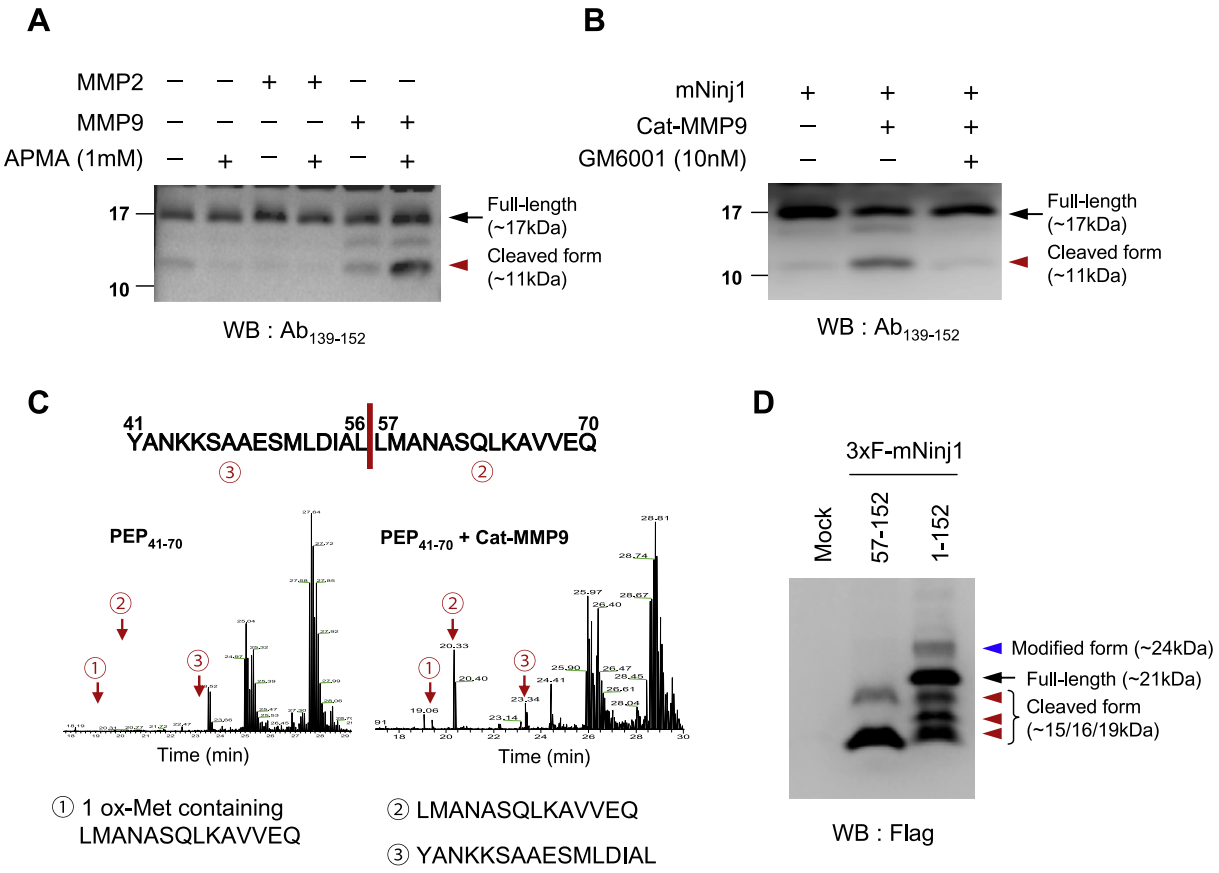

Fig. 3. MMP9-mediated Ninjurin1 cleavage between Leu^{56} and Leu^{57}. (A) The lysates from non-tagging mNinj1-overexpressed HEK293T cells were immunoprecipitated with Ab_{1-15} antibody and incubated with recombinant MMP2 or MMP9 with or without p-aminophenylmercuric acetate (APMA, 1 mM), a MMPs activator, for 16 h at 37 °C. The full-length mouse Ninjurin1 (black arrow, ~17 kDa) or its cleaved forms (red arrowhead, ~11 kDa) were identified by Western blotting for $Ab_{139-152}$ antibody. (B) The mouse Ninjurin1 protein immunoprecipitated with Ab_{1-15} antibody from the non-tagging mNinj1 transfectants was incubated with truncated MMP9 with catalytic domain (Cat-MMP9). The cleavage of Ninjurin1 mediated Cat-MMP9 was prevented by incubation with GM6001 (10 nM), a pan-MMP inhibitor. (C) PEP_{41-70} corresponding to aa 41–70 on mouse $Ninjurin1_{1-70}$ was synthesized. The incubation of PEP_{41-70} with MMP9 generated three additional peaks: ①, ②, and ③. MS analysis identified each peak as the cleaved fragments of PEP_{41-70} and its modified forms: ② matched with PEP_{57-70} fragment, ① matched with the oxidized-Methionine of PEP_{57-70}, and ③ matched with PEP_{41-56}, which is the remaining portion of PEP_{57-70}. (D) The 3×Flag-$mNinj1_{57-152}$, the remaining portion of the full-length 3×Flag-mNinj1 after cleavage, was transfected into HEK293T cells for 24 h. The 3×Flag-$mNinj1_{57-152}$ generated a product corresponding to the smallest one (~15 kDa) among cleaved produces from the 3×Flag-mNinj1. The full-length 3×Flag-mNinj1 (~21 kDa), its cleaved forms (~15/16/19 kDa), or another modified one (~24 kDa) are shown with the black arrow, red arrowheads, or blue arrowhead, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4A). These results suggest that the soluble fragments of Ninjurin1 might have chemokine-like activity.

To prove the chemokine-like role of the cleaved Ninjurin1 fragment, a trans-well assay was performed. Since the GFP-mNinj1 transfectants contain the cleaved fragments in CM (Fig. 1D), we examined the migration activity of Raw264.7 cells, mouse peritoneal macrophage cells, toward GFP or GFP-mNinj1 CM. Interestingly, the migration of Raw264.7 cells toward the GFP-mNinj1 CM was higher than that toward the GFP CM, and moreover, their activity was significantly decreased by neutralization of GFP-mNinj1 CM with Ab_{1-15} antibody treatment (Fig. 4B and C). This result indicates that the liberated N-terminal ectodomain of Ninjurin1 could have a chemotactic activity.

4. Discussion

In this study, we demonstrated the cleavage of Ninjurin1 not only in a vector system *in vitro* but also in mouse tissues *in vivo*, particularly, in the liver and kidneys. Similar to the previous results in *Drosophila* [9], MMP9 is responsible for the cleavage of mouse Ninjurin1 between Leu^{56} and Leu^{57} in its N-terminal ectodomain. Intriguingly, the liberated ectodomain of Ninjurin1 seems to have a chemotactic activity that is supported by the secondary structure similarity with well-known chemokines.

According to our computational predictions, the liberated fragment of Ninjurin1 satisfies the structural prerequisites for a chemokine-like behavior. For the general structure of chemokines, the N-terminal disordered regions can interact with the chemokine receptors to contribute as a key signaling domain. The β-sheet and C-terminal α-helix domain can serve as binding determinants with themselves for oligomerization or with glycosaminoglycans (GAGs) for presentation on the surface of endothelial cells [15,20,21]. Interestingly, all of such structural elements are predicted in the liberated fragment of Ninjurin1. Furthermore, some chemokines act as a dimer or oligomer [15]. Since Ninjurin1 binds through the homophilic binding domain, from Ala^{26} to Val^{37} [2], it is possible for the librated fragment of Ninjurin1 to dimerizes or oligomerizes with itself. Collectively, these computational studies support the structural prerequisites for the soluble fragment of Ninjurin1 to hold a chemokine-like property. In the future, structural studies like X-ray crystallography or NMR analysis on the liberated Ninjurin1 fragments will be helpful in elucidating the structure–function relationships.

Besides the shedding between Leu^{56} and Leu^{57}, other cleavage events might exist in front of the Leu^{56} residue and thereby produce fragments corresponding with the ~16 kDa and ~19 kDa fragments of the 3×Flag-mNinj1 transfectants (Fig. 1C) as well as the ~12 kDa and ~15 kDa fragments of the mouse kidneys

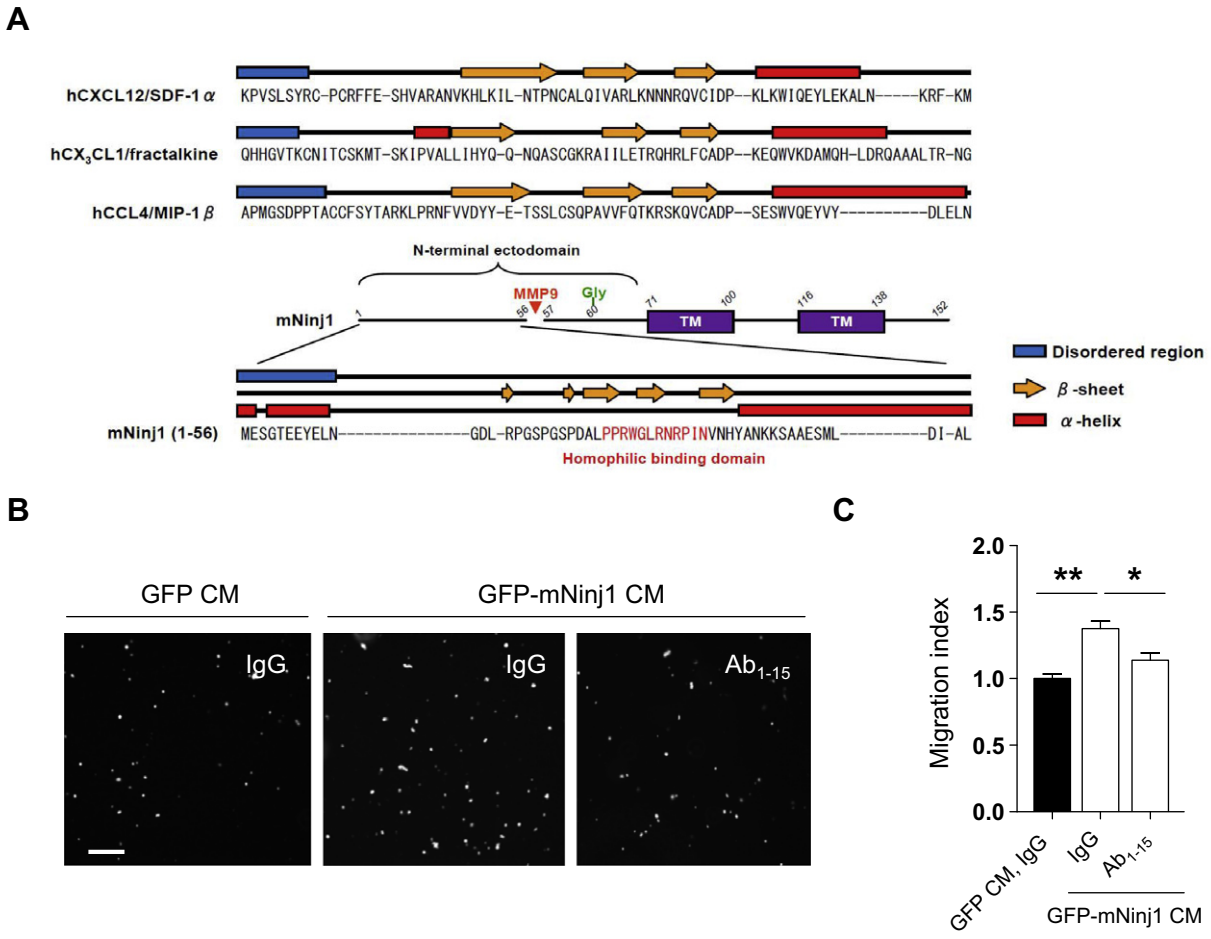

Fig. 4. The fragment of Ninjurin1 with its chemokine-like structure can attract Raw264.7 cells. (A) The structural similarity of mNinj1$_{1-56}$ and well-known chemokines. The disordered region, β-sheet, and α-helix are shown with blue rectangles, yellow arrows, and red rectangles, respectively. Human SDF-1α, fractalkine, MIP-1β, and mNinj1$_{1-56}$ were aligned by the program T-Coffee software [19]. The structural information of each chemokines was obtained from previous NMR studies [16–18]. The structure of mNinj1$_{1-56}$ was predicted with bioinformatics software programs: Garnier–Osguthorpe–Robson method for secondary structure, DisEMBL for the disordered region, and NetNGly 1.0 for N-glycosylation site. TM; Transmembrane domain, Gly; N-glycosylation. (B and C) The migration activity of Raw264.7 cells toward the liberated GFP-mNinj1 fragment. Raw264.7 cells labeled with CSFE (5 μM, 5 min) were added to the upper chamber of the trans-well and the CM from GFP or GFP-mNinj1 overexpressed-HEK293T cells in the slower chamber. GFP-mNinj1 CM was neutralized by incubation with either IgG isotype or Ab$_{1-15}$ antibody (10 μg/ml). After 8 h, the trans-well was fixed with 4% PFA and its upper side was cleaned using cotton, after which it was mounted on a glass slide. The migrated cells were visualized under microscopy. Representative images are shown in (B), and the migration index ($n = 3$) (C) is shown as a relative value to the GFP CM with IgG: $^{*}p < 0.05$, $^{**}p < 0.01$, Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2B). Furthermore, as shown in Figs. 1C and 3D, one additional band (~24 kDa, blue arrowhead) above the full-length Ninjurin1 was observed in the 3×Flag-mNinj1 transfectants, indicating the presence of other posttranslational modifications like glycosylation. Indeed, N-glycosylation on the Asn^{60} residues of mouse Ninjurin1 is predicted by NetNGlyc 1.0, a software program that predicts glycosylation. Therefore, it would be worthwhile to study additional cleavage events of Ninjurin1 or other posttranslational modifications and their pathophysiological roles.

Our study and other previous studies in the EAE animal model and in Multiple Sclerosis patients clearly demonstrate the contributions of Ninjurin1 on leukocyte trafficking and provide the feasibility for its clinical applications [3–5]. Therefore, the role of MMP9-mediated cleavage of Ninjurin1 and its chemotactic activity in disease conditions should be further explored to find strategies for modulating Ninjurin1-dependent immune responses.

Acknowledgments

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References

[1] T. Araki, J. Milbrandt, Ninjurin, a novel adhesion molecule, is induced by nerve injury and promotes axonal growth, Neuron 17 (1996) 353–361.
[2] T. Araki, D.B. Zimonjic, N.C. Popescu, J. Milbrandt, Mechanism of homophilic binding mediated by ninjurin, a novel widely expressed adhesion molecule, J. Biol. Chem. 272 (1997) 21373–21380.
[3] B.J. Ahn, H.J. Lee, M.W. Shin, J.H. Choi, J.W. Jeong, K.W. Kim, Ninjurin1 is expressed in myeloid cells and mediates endothelium adhesion in the brains of EAE rats, Biochem. Biophys. Res. Commun. 387 (2009) 321–325.
[4] I. Ifergan, H. Kebir, S. Terouz, J.I. Alvarez, M.A. Lecuyer, S. Gendron, L. Bourbonniere, I.R. Dunay, A. Bouthillier, R. Moumdjian, A. Fontana, A. Haqqani, A. Klopstein, M. Prinz, R. Lopez-Vales, T. Birchler, A. Prat, Role of Ninjurin-1 in the migration of myeloid cells to central nervous system inflammatory lesions, Ann. Neurol 70 (2011) 751–763.

[5] F. Odoardi, C. Sie, K. Streyl, V.K. Ulaganathan, C. Schlager, D. Lodygin, K. Heckelsmiller, W. Nietfeld, J. Ellwart, W.E. Klinkert, C. Lottaz, M. Nosov, V. Brinkmann, R. Spang, H. Lehrach, M. Vingron, H. Wekerle, C. Flugel-Koch, A. Flugel, T cells become licensed in the lung to enter the central nervous system, Nature 488 (2012) 675–679.
[6] J. Cichy, E. Pure, The liberation of CD44, J. Cell Biol. 161 (2003) 839–843.
[7] D.M. Smalley, K. Ley, L-selectin: mechanisms and physiological significance of ectodomain cleavage, J. Cell Mol. Med. 9 (2005) 255–266.
[8] T. Imai, K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T.J. Schall, O. Yoshie, Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion, Cell 91 (1997) 521–530.
[9] S. Zhang, G.M. Dailey, E. Kwan, B.M. Glasheen, G.E. Sroga, A. Page-McCaw, An MMP liberates the Ninjurin A ectodomain to signal a loss of cell adhesion, Genes Dev. 20 (2006) 1899–1910.
[10] H.J. Lee, B.J. Ahn, M.W. Shin, J.W. Jeong, J.H. Kim, K.W. Kim, Ninjurin1 mediates macrophage-induced programmed cells death during early ocular development, Cell Death Differ. (2009).
[11] J. Verspurten, K. Gevaert, W. Declercq, P. Vandenabeele, Sitepredicting the cleavage of proteinase substrates, Trends Biochem. Sci. 34 (2009) 319–323.
[12] A.J. Barrett, Bioinformatics of proteases in the MEROPS database, Curr. Opin. Drug Discovery Dev. 7 (2004) 334–341.
[13] J. Garnier, D.J. Osguthorpe, B. Robson, Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins, J. Mol. Biol. 120 (1978) 97–120.
[14] R. Linding, L.J. Jensen, F. Diella, P. Bork, T.J. Gibson, R.B. Russell, Protein disorder prediction: implications for structural proteomics, Structure 11 (2003) 1453–1459.
[15] S.J. Allen, S.E. Crown, T.M. Handel, Chemokine: receptor structure, interactions, and antagonism, Annu. Rev. Immunol. 25 (2007) 787–820.
[16] M.P. Crump, J.H. Gong, P. Loetscher, K. Rajarathnam, A. Amara, F. Arenzana-Seisdedos, J.L. Virelizier, M. Baggiolini, B.D. Sykes, I. Clark-Lewis, Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1, EMBO J 16 (1997) 6996–7007.
[17] L.S. Mizoue, J.F. Bazan, E.C. Johnson, T.M. Handel, Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1, Biochemistry 38 (1999) 1402–1414.
[18] S. Kim, S. Jao, J.S. Laurence, P.J. LiWang, Structural comparison of monomeric variants of the chemokine MIP-1beta having differing ability to bind the receptor CCR5, Biochemistry 40 (2001) 10782–10791.
[19] P. Di Tommaso, S. Moretti, I. Xenarios, M. Orobitg, A. Montanyola, J.M. Chang, J.F. Taly, C. Notredame, T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension, Nucleic Acids Res. 39 (2011) W13–17.
[20] H.J. Dyson, P.E. Wright, Intrinsically unstructured proteins and their functions, Nat. Rev. Mol. Cell Biol. 6 (2005) 197–208.
[21] A. Mantovani, R. Bonecchi, M. Locati, Tuning inflammation and immunity by chemokine sequestration: decoys and more, Nat. Rev. Immunol. 6 (2006) 907–918.