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The production of soluble C-type lectin-like receptor 2 is a regulated process

Min Fei · Lei Zhou · Jianhui Xie · Yuanyuan Ruan · Jiejie Xu · Songbin He · Hongjie Shen · Yumin Hu · Shifang Ren · Changgeng Ruan

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Abstract C-type lectin-like receptor 2 (CLEC-2) is a newly identified type II transmembrane protein belonging to the C-type lectin family molecules, which acts as a cell-surface receptor for snake venom toxin rhodocytin and tumor antigen podoplanin. We previously demonstrated that the full-length mouse CLEC-2 (mCLEC-2) can be cleaved into soluble form. Elevated levels of soluble forms of membrane proteins in circulating blood may reflect increased expression of membrane proteins and disease activities. In the present study, we clarified the domain and sites contributing to the production of soluble mCLEC-2. The shedding process can be positively regulated by protein kinase C (PKC). Moreover, we explored the possibility that human CLEC-2 (hCLEC-2) may also be proteolyticly cleaved and released as a soluble form. We have observed that the production of soluble hCLEC-2 could be induced by phorbol ester (PMA) in cells stably transfected with hCLEC-2 cDNA. Further studies may explore therapeutic and diagnostic applications of soluble hCLEC-2 in platelet-related diseases.

Keywords CLEC-2 · Soluble form · PKC · Phorbol ester

Min Fei, Lei Zhou, Shifang Ren and Changgeng Ruan contributed equally to this work.

M. Fei · H. Shen · C. Ruan
Jiangsu Institute of Hematology, Soochow University, Suzhou, Jiangsu, China

M. Fei · C. Ruan
Institute of Medical Biotechnology, Soochow University, Suzhou, China

L. Zhou · J. Xie · Y. Ruan · J. Xu · S. Ren (✉)
Department of Biochemistry, Shanghai Medical School, Fudan University,
Shanghai, 200032, People's Republic of China
e-mail: renshifang@fudan.edu.cn

S. He
Department of General Surgery,
The First Affiliated Hospital of Soochow University, Suzhou, China

Y. Hu
Institute of Medical Biotechnology, Soochow University, Suzhou, China

C. Ruan (✉)
Jiangsu Institute of Hematology,
The First Affiliated Hospital of Soochow University, Suzhou, China
e-mail: changgengruan@yahoo.com.cn

Introduction

C-type lectin-like receptor 2 (CLEC-2) was original identified as a type II transmembrane protein with a short N-terminal cytoplasmic tyrosine-based domain and a single carbohydrate recognition domain, whose structure belongs to the C-type lectin family [1]. The transcripts of CLEC-2 have been shown in the liver and myeloid cells, while the proteins are expressed primarily on the surface of platelets as dimers [2]. Clustering of CLEC-2 by the snake venom toxin rhodocytin leads to the activation of platelets in syk-dependent manner [3]. CLEC-2 is reported to be the physiological counterpart for podoplanin, and their interaction may be involved in podoplanin-induced platelet aggregation, tumor metastasis, and other cellular responses [4, 5]. In addition, CLEC-2 has been identified as an attachment factor for human immunodeficiency virus type 1(HIV-1), and facilitates the capture and transfer of HIV-1 by platelets [6]. The receptor has also been found as an activation receptor on neutrophils [7]. Recently, we reported RACK1 associated with the cytoplasmic tail of CLEC-2, and

decreased the stability of CLEC-2 through promoting its ubiquitin-proteasome degradation [8].

We previously identified two splice variants of murine CLEC-2 which had different expression profiles and subcellular localization from the full-length form. Moreover, the full-length mCLEC-2 can be cleaved into soluble form [9]. In the past, a number of cell receptors have been found to be proteolytically cleaved at their juxtamembrane region, resulting in detachment of the extracellular region. Elevated levels of soluble forms of membrane proteins in circulating blood in humans may reflect increased expression of membrane proteins and disease activities. In addition, soluble forms of receptors may interact with ligands, inhibit the binding of ligands to the cell-surface receptor, and thus modulate the pathophysiology [10–12].

In the present study, therefore, we tried to gain insight into details of mCLEC-2 cleavage, and explore the possibility that human CLEC-2 (hCLEC-2) may also be proteolyticly cleaved and released as soluble forms. We have clarified the domain and sites contributing to the production of soluble mCLEC-2. Furthermore, we have observed that the production of soluble hCLEC-2 could be induced by activating PKC.

Material and methods

Cell lines and culture conditions Chinese hamster ovary (CHO) cells were grown in Ham's F12 medium. Human embryonic kidney 293T cells and breast cancer MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). The culture media were purchased from Sigma, supplemented with 10 % fetal calf serum (Gibco) and 50 U/ml streptomycin/penicillin (Sigma-Aldrich).

Plasmid construction To construct the plasmid that expressed human CLEC-2 for stable transfection, the coding region of human CLEC-2 (GenBank Accession No. NM_016509.3) was amplified by PCR using a human liver cDNA library (Clontech) as the template. The product was cloned into the vector pIRES2-EGFP (Invitrogen), which contains the internal ribosome entry site between the MCS and EGFP coding region, thus both the gene of interest and the EGFP gene would be translated from a single bicistronic mRNA.

Site-directed mutagenesis Mutagenesis was performed by overlap extension using PCR and appropriate primers for site-directed mutagenesis are as follows:

HSM:

F: 5′-GCTCTGGGGATCATGTCGGTCACACAG C A A A A G T A T C T A C T G G C G G A G A A G GAAAATCTCTCAGCGACTCTGCAAC-3′

R : 5′-G T T G C A G A G T C G C T G A G A G A T T T T C C T T C T C C G C C A G T A G A TACTTTTGCTGTGTGACCGACATGATCCCCA GAGC-3′

mCLEC-2-K59, 65G:

59K/G:

F: 5′-CTGTCATGCAGAGCAATTACC-3′
R: 5′-GGTAATTGCTCTGCATGACAG-3′

65K/G:

F: 5′-GGTGAGAATGAAAATAGCACAGGAAC-3′
R :5′-GTTCCTGTGCTATTTTCATTCTCACC-3′

Western blotting Cells were lysed, separated on SDS-PAGE gels and transferred to PVDF membranes as previously described. Immunoblotting was performed as previously described with the addition of indicated antibodies. Soluble CLEC-2 was purified using Ni-NTA resin (QIAGEN) according to the manufacturer's instructions.

Immunofluorescence Microscopy Cells on the glass cover slips were fixed with ice-cold methanol. Briefly, cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min at room temperature. After rinsed three times with PBS, the nonpermeabilized cells were detected for cell surface molecules.

Flow cytometry analysis Stably transfected MCF-7 cells were first incubated with mouse anti-myc primary antibody for 1 h at 4 °C, washed three times with ice-cold PBS, and subsequently incubated with FITC-labeled secondary antibody for another 30 min at 4 °C. After extensive wash, cells were suspended in PBS/1 % BSA and subjected to FACS for analysis

Results

The cleavage sites of mouse CLEC-2 are located at the carboxyl side of lysine in the neck region

The C-type lectin-like receptor CLEC-2 was identified as a type II transmembrane protein without signal peptide. The CTLD domain in CLEC-2 is supported by a 41 amino acid neck region, a single transmembrane domain and a 31 amino acid cytoplasmic domain [13]. In the previous study, we observed that full-length mouse CLEC-2 (mCLEC-2) could be cleaved into a soluble form that partially existed as a disulfide-linked homodimer. Two serine protease inhibitors, aprotinin and phenylmethyl sulfonylfluoride (PMSF) reduced the release

of soluble mCLEC-2 about 70 % and 40 %, respectively. These observations indicated that soluble mCLEC-2 is released by proteases sensitive to aprotinin and PMSF [9]. Aprotinin is a monomeric globular polypeptide derived from bovine lung tissue, which inhibits several serine proteases, specifically trypsin [14, 15]. We speculated the cleavage of mCLEC-2 might be associated with trypsin. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine [16]. Based on the observation, we analyzed the amino acids sequence in neck region, and found that there were two amino acids at Lys59 and Lys65 respectively, which were possibly sensitive to serine protease. Given that CLEC-2 has a similar structure with LOX-1, we compared their amino acids sequence in neck domain (Fig. 1a). In TNF-α activated cells, surface expression of LOX-1 precedes soluble LOX-1 production, which was inhibited by PMSF. Purification of soluble LOX-1 by high-performance liquid chromatography and N-terminal amino acid sequencing of soluble LOX-1 identified the 2 cleavage sites between Arg86-Ser87 and Lys89-Ser90 [17]. This result is consistent with our speculation to the cleavage sites of mCLEC-2.

To test the hypothesis, a mutant construct ((K59, 65G) was generated. Conditioned media of CHO-K1 cells transfected with myc-tagged variants were purified, followed by western blotting. As Fig. 1b depicted, mutation of Lys59, 65 thoroughly kept mCLEC-2 from forming soluble form, confirming the speculation that these two sites might play a significant role in the cleavage of mCLEC-2.

Production of the cleavage of mCLEC-2 is enhanced by the activation of protein kinase C

Protein ectodomain shedding occurs at or near the cell surface, which can be activated by different mechanisms. This is exemplified by sLOX-1, which is induced in the stimulation of TNF-α. Use of phorbol esters (PMA), well characterized nonphysiological compounds with the ability to activate protein kinase C (PKC), is the most common way to activate ectodomain shedding [18, 19]. To examine whether the production of soluble mCLEC-2 could be activated by stimulatory factors, cell-conditioned media and whole-cell lysates from CHO-K1 cells transfected with mCLEC-2 cDNA cultured in the presence of DMSO(4 μl/ml,as negative control), TNF-α(10 ng/ml)and PMA(300 ng/ml, respectively,) for indicated time were subjected to western blotting. Figure 2a demonstrates that the production of soluble mCLEC-2 in cultured medium was enhanced by PMA treatment. In TNF-α treated cell, in contrast, no obvious change was detected in the protein in conditioned medium.

The results suggest that the PKC pathway may participate in the shedding process of mCLEC-2. To further corroborate the results, we tested the effects of two PKC pathway inhibitors Gö6976 (Sigma) and CGP 41251 (Sigma) on the release of soluble form from transfected CHO-K1 cells, respectively. As shown in Fig. 2b and 2C, both inhibitors reduced the release of soluble mCLEC-2 compared to the protein in cell lysates. These observations indicated that PKC pathway positively regulates the shedding process of mCLEC-2.

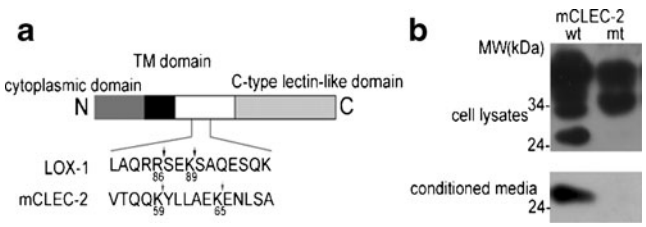

Fig. 1 Site-mutation at Lys 59 and Lys 65 abolish the production of soluble mouse CLEC-2. **a** Scheme of the predicted cleavage sites of mCLEC-2 and the 2 cleavage sites of LOX-1. The transmembrane domain is indicated by the solid box, and the C-type lectin-like domain is in light gray. Arrows show the (predicted) cleavage sites. **b** CHO-K1 cells were tranfected with wild type or mutated mCLEC-2 (K59, 65G) for 8 hours, and then cultured in serum-free medium for another 24 hours before harvested. Cell lysates and supernatants were subjected to western blotting by detecting with anti-myc antibody

Replacement of neck region causes shedding of human CLEC-2

Ectodomain shedding of protein is an important way to regulate receptor function, which is best exemplified by platelet surface receptors, such as GPIb-IX-V complex, G, PECAM-1, and Sema4D. Cell receptors can be proteolytically cleaved at their juxtamembrane region, resulting in detachment of their extracellular region. Shedding can release cytokines and down-regulate receptor function. Mean while, several released soluble receptors have been discovered in human circulating blood, which could be useful markers for early diagnosis of some diseases [20–22]. Given that the newly identified platelet receptor CLEC-2 is cleaved into soluble form in mouse, we attempted to test whether human CLEC-2 (hCLEC-2) exists as a soluble form. CHO-K1 cells were transfected with myc-tagged hCLEC-2 plasmid, and then cell lysates and conditioned medium were prepared. Western blotting analysis showed that different from mCLEC-2, hCLEC-2 was undetectable in culture medium. We also transfected the plasmid into COS-7 and HEK-293T cells, and analyzed the CLEC-2 in the culture medium. Similar results were observed to those obtained with CHO cells. To address this question, we analyzed the amino sequences of mCLEC-2 and hCLEC-2. The full-length mCLEC-2 gene has 62.6 % homology with hCLEC-2 gene, while the two original proteins share lower homology in neck region (44.4 %). Therefore, we substituted the neck region of mCLEC-2 (53aa-70aa) for hCLEC-2 (54aa-71aa), and constructed chimeric hCLEC-2/mCLEC-2

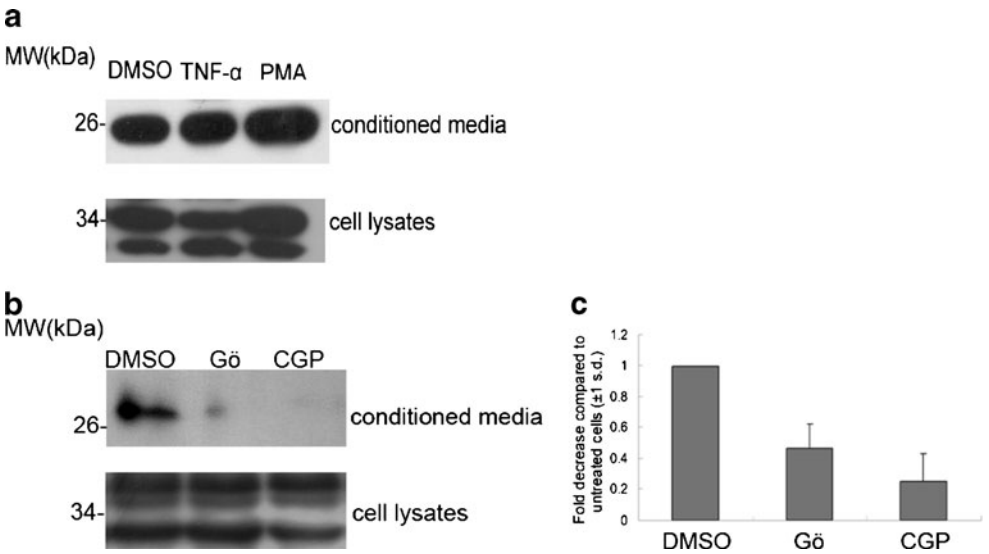

Fig. 2 PMA positively regulates the process of mouse CLEC-2 shedding. **a** Immunoblotting with anti–myc mAb in whole-cell lysates and conditioned media of mCLEC-2 transfected CHO-K1 cells. Transfected cells were treated with DMSO(4 μl/ml),TNF-α(10 ng/ml)and PMA(300 ng/ml)for 8 hours, respectively. Equal protein purified from cell-conditioned media and whole-cell lysates were subjected to immunoblotting with anti–myc mAb. **b** Transfected CHO-K1 cells were incubated with 4 μm Gö6976 and CGP 41251 for 8 hours, respectively. Cell lysates and supernatants were subjected to western blotting by detecting with anti-myc antibody. **c** Fold decrease in shedding of mouse CLEC-2 compared to DMSO treated cells. The ratios of soluble mouse CLEC-2 to the expression level of mouse CLEC-2 were calculated using ToalLab software program. The value from DMSO treated cells was set as 1. The values from at least three independent experiments were used to calculate. Values were plotted ± 1SD

plasmid nominated HSM (Fig. 3a). CHO-K1 cells were transfected with the chimeric plasmid, followed by western blotting analysis. HSM protein fragment was detectable in the cultured medium (Fig. 3b), which means that the neck region of mCLEC-2 is more sensitive to proteolysis compared to hCLEC-2.

Production of soluble hCLEC-2 is induced by activators of protein kinase C

Previous study indicated that, unlike mCLEC-2, no soluble hCLEC-2 was detected in cultured medium in untreated cells. Meanwhile, activation of PKC pathway positively regulates the production of soluble mCLEC-2. To test the hypothesis that hCLEC-2 may also be converted into soluble molecules in the activation of PKC, myc-tagged hCLEC-2 fragment was cloned into the vector pIRES2-EGFP (Invitrogen), which contains the internal ribosome entry site between the MCS and EGFP coding region. The plasmid was stably transfected into MCF-7 cells. Cell surface expression of the protein was confirmed by immunofluorescence and flow cytometry analysis (Fig. 4a, b). Same amount of stably transfected cells were cultured with DMSO (4 μl/ml, as negative control), TNF-α (10 ng/ml) and PMA (300 ng/ml) for indicated time in serum-free medium. As shown by the western blotting of cell lysates and cultured medium, the expression of soluble hCLEC-2 was undetectable in DMSO treatment. In response to PMA, soluble hCLEC-2 was detectable, while the expression of cell-surface hCLEC-2 was slightly increased. Conversely, TNF-α treatment had no effect on the expression, consistent with

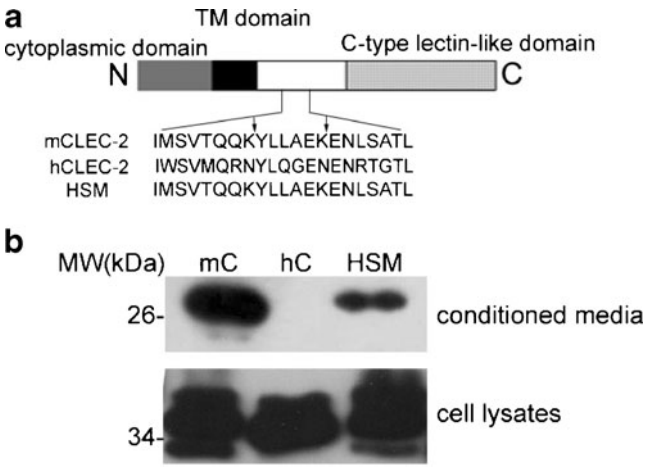

Fig. 3 Amino acid sequence in stalk region contributes to the shedding of CLEC-2. **a** Scheme of stalk region of mCLEC-2 and hCLEC-2. The HSM Chimera was generated by PCR, such that the chimeric sequence translated as hCLEC-2 1–53/mCLEC-2 53-70/hCLEC-2 72-229 generating a chimeric receptor consisting of the stalk region of hCLEC-2 and the CRD, trasmembrane, and cytoplamic tail of mCLEC-2. The transmembrane domain is indicated by the solid box, and the C-type lectin-like domain is in light gray. Arrows show the cleavage sites. **b** CHO-K1 cells were tranfected with mCLEC-2, hCLEC-2 and HSM plasmid, respectively. Six hours after transfection, the cells were cultured in serum-free media for another 12 hours. Cell lysates and purified supernatants were subjected to western blotting by detecting with anti-myc antibody

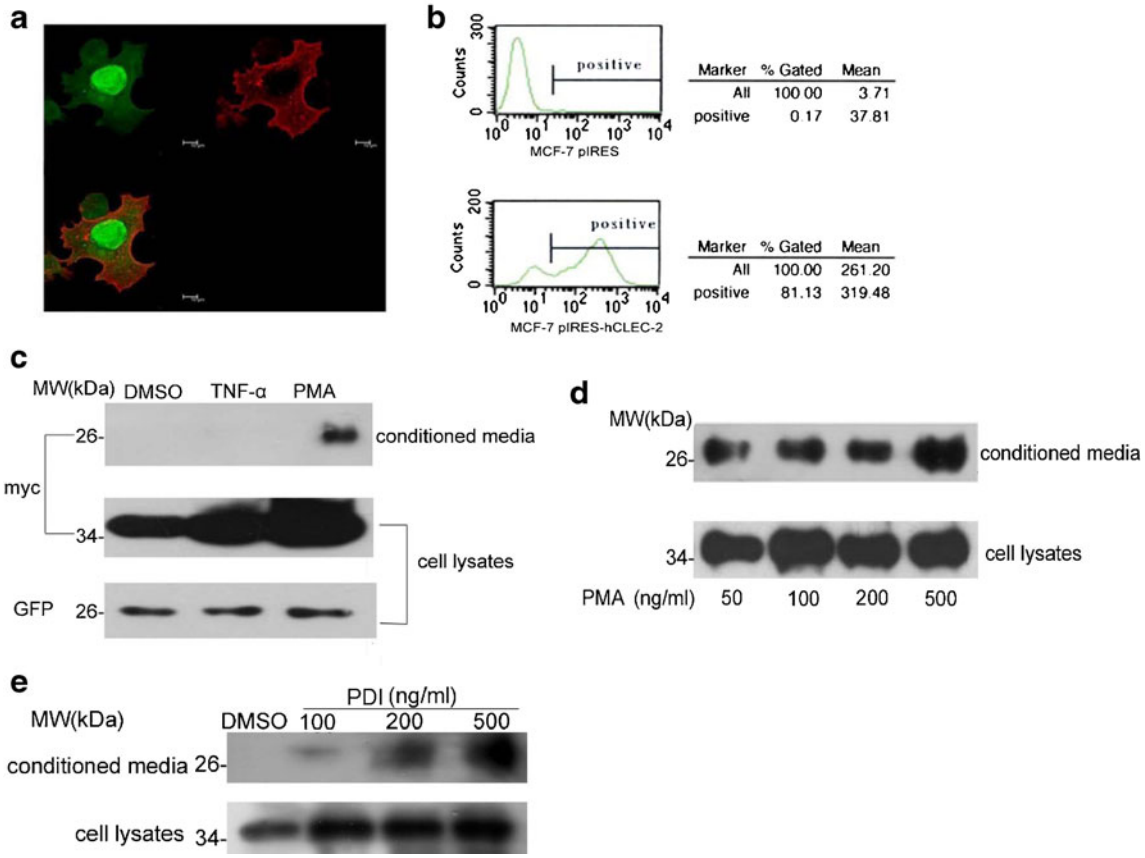

Fig. 4 Activation of PKC pathway induces the production of soluble human CLEC-2 fragment. **a** Confocal microscopic analysis of the location of human CLEC-2 in MCF-7 stable transfectants. MCF-7 cells stably transfected with human CLEC-2 were fixed, and then incubated with mouse anti-myc monoclonal antibody and fluorescein conjugated secondary antibodies (anti-mouse IgG-Rhodamine). The sub-cellular localization of hCLEC-2 (red) and EGFP (green) was analyzed by confocal laser scanning fluorescence microscope. **b** MCF-7 cells expressed myc-tagged hCLEC-2 were counted by flow cytometry analysis. Cells tranfected with pIRES-EGFP vector were set as control. **c** Transfected MCF-7 were treated with DMSO(4 μl/ml),TNF-α(10 ng/ml)and PMA (300 ng/ml)for 8 hours, respectively. Equal protein purified from cell-conditioned media and whole-cell lysates were subjected to immunoblotting with anti–myc mAbs and anti-GFP antibody. **d**, **e** Transfected MCF-7 cells were treated with different dose of PMA and PDI for 8 hours, respectively. Equal protein purified from cell-conditioned media and whole-cell lysates were subjected to immunoblotting with anti–myc mAbs

previous results (Fig. 4c). Furthermore, we observed that the protein concentration of soluble fragment was increased when the cells were treated with more PMA (Fig. 4d). Similarly, when cells were treated with PDI, an active analog of PMA, the soluble form of hCLEC-2 could also be detected. PDI treatment induced the production of soluble hCLEC-2 in a dose-dependent manner (Fig. 4e). These data demonstrate that activation of PKC pathway can induce the production of soluble hCLEC-2.

Discussion

The release of the extracellular domain through limited proteolysis has been recognized as a general mechanism to regulate the function of transmembrane proteins. This type of limited proteolysis is currently known as ectodomain shedding, affects a surprisingly large group of transmembrane proteins [23, 24]. Ectodomain shedding regulates most cellular functions, including cell adhesion, signal transduction and certain pathologies such as Alzheimer's disease or cancer [25].

Ectodomain shedding occurs at or near the cell surface and is a regulated process. It can be dramatically activated by several independent mechanisms, although it occurs in nonstimulated cells [26]. Using PMA to activate PKC pathway, is the most common way to activate shedding process [27]. In our experiments, we demonstrated that similar to other transmembrane proteins, activation of PKC pathway could enhance the ectodomain shedding of mCLEC-2 and induce the production of soluble hCLEC-2. We also clarified the cleavage sites of mCLEC-2 and compared the sensitivity to shedding process of the neck regions from mCLEC-2 and hCLEC-2. Previous researches indicated that PKC activates the shedding of many different proteins [27]. Investigation of the intracellular signaling pathways that up-regulate shedding revealed that although several

independent pathways seem to be involved in regulating ectodomain shedding, mitogen-activated protein (MAP) kinases are common regulators of the shedding process [28]. Further studies may explore the exact role of PKC signal pathway in the shedding of CLEC-2.

CLEC-2 is a newly identified C-type lectin-like receptor and plays an important role in platelet activation. The platelet plasma membrane is literally at the cutting-edge of recent research into proteolytic regulation of the function and surface expression of platelet receptors, revealing new mechanisms for how the thrombotic propensity of platelets is controlled in health and disease. Extracellular proteolysis of receptors irreversibly inactivates receptor mediated adhesion and signaling, as well as releasing soluble fragments into the plasma where they act as potential markers or modulators [29]. Plasma concentrations of such receptors are correlated with the levels of their counterparts on the cell surface and probably reflect a certain disease status *in vivo*. This is exemplified by the recent reports that sLOX-1, which derives from proteolytic cleavage of membrane-bound LOX-1, appears to be a useful marker for early diagnosis of acute coronary syndrome [30]. In the present study, we demonstrated that hCLEC-2 could be truncated into soluble form induced by PKC activation, indicating the potential function of soluble hCLEC-2. Besides, these results indicated that suitable antibodies might be useful tools for detecting the soluble CLEC-2 in plasma, and might have clinical applications. Further studies may focus on the production of proper antibodies for preclinical studies using animal models, and explore therapeutic and diagnostic applications of soluble CLEC-2 in platelet-related diseases.

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