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ORIGINAL ARTICLE

Functional expression of the Fc-fused extracellular domains of group II membrane proteins

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Abstract The complicated delivery mechanism of group II membrane proteins makes it difficult to decide the fusion pattern of their extracellular domains (ECDs) with Fc moiety. In this study, we compared the expression of ECDs of three group II membrane proteins including CLEC-2, Dectin-1, and LOX-1 by fusion of Fc moiety. We found that the pattern of ECD-Fc fusion order produced the functionally active recombinant proteins while the pattern of Fc-ECD fusion order led to the altered glycosylation which abolished the binding of these proteins with their ligands. Meanwhile, our results indicated that the secretion of mouse Fc (mFc)-fused ECD of CLEC-2 was more efficient than that of rabbit Fc (rFc)-fused protein, while rFc moiety was more sensitive for detection compared with mFc moiety. Altogether, we provide a favorable fusion pattern of Fc moiety with the ECDs of group II transmembrane proteins.

Keywords Fc moiety · Extracellular domain · Group II membrane proteins · CLEC-2

Introduction

There are a number of transmembrane proteins expressed on the cell surface. Generally, these transmembrane proteins are divided into three groups based on their orientation in the membrane [1]. Their cytoplasmic tails and the transmembrane regions are thought to function as internal signal sequences with a crucial role in the delivery to the cell surface. Among these proteins, Group I membrane proteins, such as epidermal growth factor receptor (EGFR), have a cleavable N-terminal signal peptide [2], while Group II membrane proteins, such as transferrin receptor, lack the peptide [3]. Many of these transmembrane proteins are receptors and play vital roles in the

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maintenance of homeostasis. To clarify their functions, the identification of their endogenous and exogenous ligands is necessary and the recombinant products of their extracellular domains (ECDs) are very useful.

Due to the functional glycosylation on the ECDs of these transmembrane receptors [4], it is a major challenge to efficiently obtain recombinant products with native folding [5]. In order to bear the carbohydrate modifications, these proteins can be expressed in eukaryotic cells including CHO-K1 cells and HEK293T cells [6]. These extracellular regions can be fused with various affinity tags to be purified from the background of total cellular proteins [7]. Most of fusion tags are developed in the last 30 years, including 6×Histidine (His6), Glutathione S-transferase (GST), and so on. Among them, the Fc moiety of immunoglobulin G (IgG) is used in diverse applications. The Fc moiety confers an intrinsic dimerization, which provides the recombinant proteins with the bivalent binding function to increase the binding avidity. The increase of the binding avidity is potent for purifying the ligands of receptors, especially for the ligands with low affinity [8]. It is convenient to fuse the ECDs of group I membrane proteins at the N-terminus of the Fc moiety, since these ECDs have a similar localization with the Fab fragment of IgG. In contrast, it is difficult to determine the fusion pattern of group II membrane proteins in that these ECDs have a similar localization with the Fc region of IgG.

Natural killer gene complex (NKC) encodes a group of C-type lectins that belong to Group II transmembrane proteins [9]. These proteins are expressed on the cell surface of NK and/or myeloid cells and play important roles in various physiological functions and pathological processes [10]. Among them, CLEC-2 has been reported to be the receptor for the platelet-aggregating snake venom toxin rhodocytin and the endogenous sialoglycoprotein podoplanin [11]. LOX-1 functions as a scavenger receptor that binds various ligands, including oxLDL, heat shock proteins, as well as C-reactive protein [12]. Dectin-1, as a non-Toll-like pattern recognition receptor (PRR), specifically recognizes β(1-3) glucans [13]. These known ligands of Group II membrane receptors can be used to determine the favorable fusion pattern of their ECDs and Fc moiety. And the fusion pattern might be suitable for other Group II proteins due to the similar structure of these proteins.

In this study, we compared the expression of the ECDs of CLEC-2, Dectin-1, and LOX-1 by fusion of Fc moiety from different species and at different locations. Our results indicated that the recombinant proteins with rabbit Fc moiety (rFc) had a more sensitive detection compared with mouse Fc moiety (mFc), although the mFc-fused proteins had a high expression yield by the evaluation of the normal IgG. Furthermore, the pattern of ECD-Fc fusion order produces the functionally active recombinant proteins with native conformation while the pattern of Fc-ECD fusion order led to the altered glycosylation and improper conformation of ECDs.

Materials and methods

Cell culture

Chinese hamster ovary (CHO-K1) cells were maintained in Ham's F12 medium. Human embryonic kidney HEK293T cells and human epithelial cervical cancer HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All culture medium was purchased from Sigma, supplemented with 10 % fetal calf serum (Gibco) and 100 U/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma). All the cells were cultured in a 5 % CO_2 atm at 37 °C. All transfections were performed with LipofectAMINE2000 (Invitrogen) according to the manufacturer's instructions.

Plasmid constructs

Restriction enzymes were purchased from Takara (Kyoto, Japan). To construct the plasmids that expressed soluble recombinant proteins, the coding sequences for ECDs of these proteins were amplified by PCR with human lung cDNA as the template. The Fc fragment was amplified by PCR with pFUSE-mIgG1 or pFUSE-rIgG1 (InvivoGen, CA) as the template. The coding sequence for the signal peptide of mouse IL-2 was added by PCR to the coding sequences of recombinant proteins at 5′-end and the products were cloned into the pcDNA3.1/Myc-His (−) vector in frame as described in Fig. 1a. The coding sequence for mouse Podoplanin was amplified by PCR and cloned into pcDNA3.1/Myc-His and pEGFP-N3, respectively. The HASPB-N18-Hsp60 plasmid was constructed as described previously [14]. All these constructs were confirmed by sequencing.

Preparation of soluble proteins

Soluble proteins were prepared as we described previously [14]. Generally, the constructs as indicated were transfected into HEK293T cells or CHO-K1 cells. After 8 h, the culture medium was replaced with the conditioned medium that was collected 30 h later and concentrated with the ultrafilter tube (10 kDa, Millipore) at 7500 g for 10 min at 4 °C. The concentrated media was dialyzed in PBS at 4 °C overnight, and the final volume of the solution was set as 1 ml polished with PBS.

His-tag pull-down

The cells transfected with or without Podoplanin construct were collected and washed with cold phosphate buffered

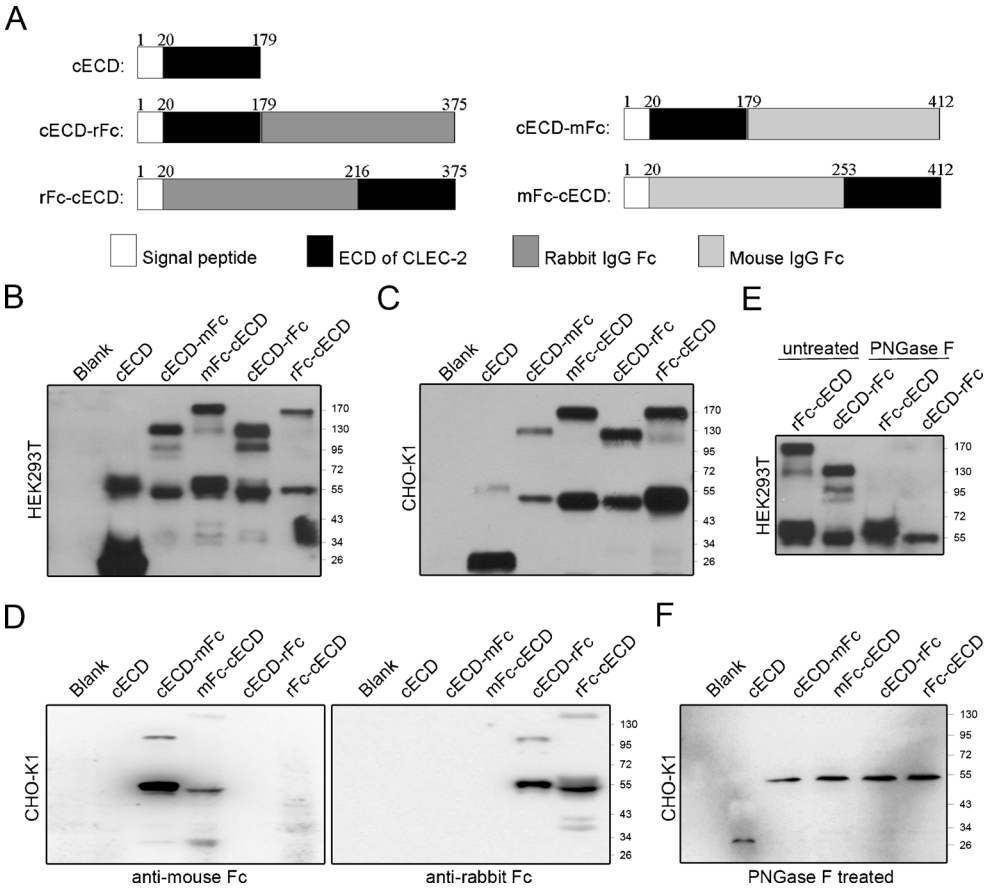

Fig. 1 Expression of Fc-fused ECD of CLEC-2 in eukaryotic cells. **a** Schematic representation of the recombinant extracellular domain (ECD) of CLEC-2 fused with the rabbit or mouse Fc fragment and the signal peptide of IL-2 in pcDNA3.1/Myc-His (−). cECD, ECD of CLEC-2; cECD-rFc, cECD fused with the rabbit Fc fragment at C-terminus; rFc-cECD, the rabbit Fc fragment fused with the signal peptide of IL-2 at N-terminus and the ECD of CLEC-2 at C-terminus; cECD-mFc, cECD fused with the mouse Fc fragment at C-terminus; mFc-cECD, the mouse Fc fragment fused with the signal peptide of IL-2 at N-terminus and the ECD of CLEC-2 at C-terminus. (**b**, **c**, **d**) Recombinant CLEC-2 from HEK293T (**b**) and CHO-K1 (**c**, **d**) with the indicated constructs. HEK293T (**b**) or CHO-K1 (**c**, **d**) cells were transfected with pcDNA3.1/Myc-His (−) vector or the indicated constructs and the conditioned culture medium was collected and concentrated under the same condition, respectively. Then, samples were subjected to western blotting with rabbit anti-mCLEC-2 antibody (**b**, **c**) or with different anti-Fc antibodies (**d**). (**e**, **f**). The conditioned culture media of HEK293T (**e**) or CHO-K1 cells (**f**) secreting recombinant CLEC-2 was concentrated and boiled followed by treatment with 5 units PNGase F at 37 °C. Samples with or without treatment were analyzed by immunoblotting probing with the HRP-conjugated antibody against rabbit Fc moiety (**e**) or anti-mCLEC-2 antibody (**f**)

saline (PBS, pH 7.4) and lysed with lysis buffer (Roche) on ice for 30 min. Lysates were clarified by centrifugation and followed by incubation with the indicated soluble proteins for 2 h at 4 °C with rotation and the immunocomplex was then mixed with 30 μl of 1:1 slurry of pre-equilibrated Ni-NTA magnetic agarose beads (Qiagen) at 4 °C overnight. After centrifugation, the pellets were washed three times with the lysis buffer containing 50 mM imidazole. The pellets were collected and subjected to Western blot analysis.

Western blot analysis

Western blot analysis was performed under reducing conditions according to Santa Cruz Biotechnology's protocol. Briefly, protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were incubated with indicated primary antibodies for 2 h at room temperature followed by the incubation with HRP-conjugated secondary antibodies. Blotted proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Tiangen Biotech). The intensity of the bands was analyzed by Quantity One V 4.62 Software and PRISM 6 software. Due to the sensitivity limits of ECL system, there are deflections in calculating high concentration proteins, which doesn't interfere the result in this study.

Deglycosylation of soluble proteins

The concentrated culture media containing soluble proteins was denatured at 100 °C for 10 min and deglycosylated using

PNGase F (Sigma) for 3 h at 37 °C, and then analyzed *via* immunoblotting.

Confocal microscopy

Cells seeded on polylysine-treated slides were transfected with podoplanin-GFP construct. After 48 h, the transfected cells were washed with PBS and fixed with 4 % paraformaldehyde for 10 min. The cells were incubated with soluble proteins at room temperature for 2 h followed by the staining of Alexa-Flour-549-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature. Cells were extensively washed with PBS and mounted on glass slides using Fluoromount mounting medium (Sigma). Labeled cells were visualized using confocal scanning microscopy (Leica Microsystems Heidelberg GmbH).

Flow-cytometric analysis

Live *Saccharomyces cerevisiae* AH109 cells were boiled for 5 min and placed on ice. 5×10^6 yeast cells were incubated with soluble Dectin-1 at 4 °C for 1 h, followed by the staining of Alexa Fluor 549-conjugated goat anti-rabbit IgG (Cell Signaling Technology) for 45 min. Subsequently, yeast cells were washed with PBS and were evaluated using a FACS Calibur flow cytometer (Beckmen).

Results

The expression of Fc-fused CLEC-2 in eukaryotic cells

Four CLEC-2 constructs with the coding sequence of mouse or rabbit Fc moiety were generated as described in materials and methods (Fig. 1a). These constructs include the ECD of CLEC-2 (cECD) fused with rFc at C-terminus (cECD-rFc) and N-terminus (rFc-cECD), respectively, and the cECD fused with mFc at C-terminus (cECD-mFc) and N-terminus (mFc-cECD), respectively. These constructs were transiently transfected into cells, respectively, and the culture medium was concentrated with ultrafilter tubes. The soluble CLEC-2 was detected by western blot with a rabbit polyclonal antibody specifically against mouse CLEC-2 or with HRP-conjugated antibodies against different Fc moieties. As shown in Fig. 1b, HEK293T cells released the soluble CLEC-2 after transfection. Two major forms of the soluble Fc-fused CLEC-2 were observed. Of note, the cECD fused with Fc moiety at the N- or C-terminus resulted in the different migration rates. Similar results were also observed in CHO-K1 cells, which implies that these two fusion patterns could result in different post-translational modifications (Fig. 1c). Furthermore, results from western blot with antibodies against mouse or rabbit Fc moiety confirmed our observation (Fig. 1d). Since C-type lectin receptors were widely reported to be modified with glycans, especially N-linked glycosylation [9, 10], we speculated that these recombinant proteins might be modified by the different degree of glycosylation. To address this issue, different recombinant CLEC-2 from transfected HEK293T or CHO-K1 cells was treated with PNGase F. As shown in Fig. 1e, PNGase F treatment resulted in about the same migration of cECD-rFc and rFc-cECD although rFc-cECD seemed to still be conjugated with other types of modification in HEK293T cells. Also, all digested recombinant CLEC-2 from CHO-K1 cells presented the same migration (Fig. 1f). In fact, results from lectin blot analysis with Erythroagglutinating phytohemagglutinin (E-PHA) revealed the different levels of bisecting GlcNAc structures on recombinant CLEC-2 with different fusion patterns (Fig. S1). Therefore, the pattern of Fc-ECD fusion order led to a significantly higher degree of N-linked glycosylation modification compared with that of ECD-Fc fusion order, which implies that the different fusion patterns of ECD and Fc render the recombinant proteins different conformations.

Expression level of cECD fused with Fc moiety from different species

We subsequently investigated the secretion efficiency of cECD fused with Fc moiety from different species. After transfection of four CLEC-2 constructs in HEK293T cells or CHO-K1 cells, the culture medium was collected and concentrated under the same condition. The equal volume of concentrated conditioned culture medium was subjected to western blot analysis with HRP-conjugated antibodies against different Fc moieties, and the quantificational mouse IgG1 and rabbit IgG were used as the standard (Fig. 2a–d). Figure 2a and c, as well as Fig. 2b and d, were treated with the same exposure condition, which ensures the detection of secretion efficiency in conformity to the actuality. Results from quantification analysis using the normal mouse or rabbit IgG (Fig. S2) showed that the release of mFc-fused CLEC-2 is more efficient than that of rFc-fused CLEC-2 in 293 T cells (Fig. 2a and b). Similar results were also observed in CHO cells (Fig. 2c and d). The different secretion efficiency of recombinant CLEC-2 was further confirmed *via* coomassie brilliant blue staining (Fig. 2e). The rFc-fused proteins presented brighter bands than mFc-fused ones, which suggest that rFc is more sensitive for detection than mFc. Therefore, the rFc-fused proteins are more sensitively detected than the mFc-fused proteins, though the secretion efficiency of rFc-fused proteins is lower than that of mFc-fused proteins.

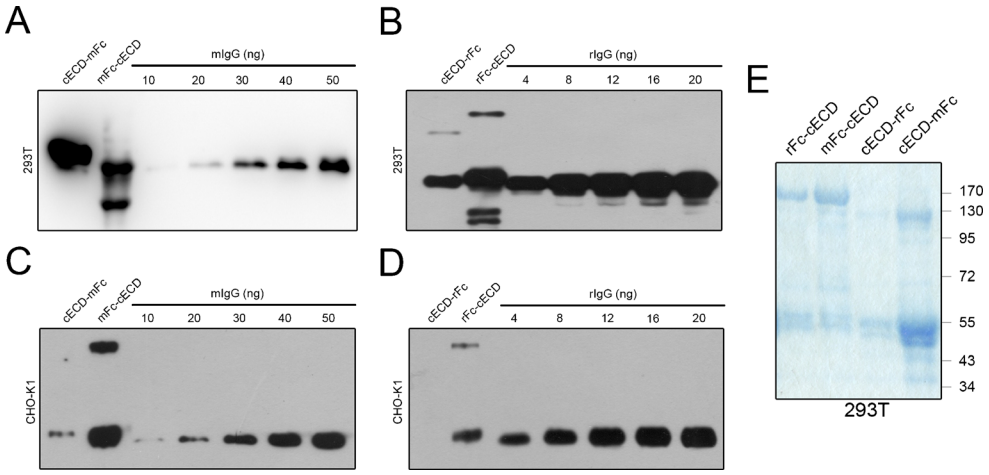

Fig. 2 Expression efficiency of different recombinant CLEC-2. (**a**-**d**) The culture medium of 293 T cells (**a**, **b**) or CHO-K1cells (**c**, **d**) transfected with the indicated constructs was collected and concentrated under the same condition. The quantificational mouse IgG1 antibody or rabbit IgG antibody were used as the standard. Equal volume of concentrated conditioned culture medium was subjected to western blot analysis. (**e**) Concentrated recombinant CLEC-2 from 293 T was analyzed *via* the coomassie brilliant blue staining. The loading volume of the concentrated media was 10 times than that in the quantificational blotting test

The pattern of cECD-Fc fusion order produces functionally active recombinant proteins

Since the rabbit Fc moiety is sensitive for detection, we used cECD fused with rFc to investigate the binding profile of CLEC-2 with podoplanin. CHO-K1 cells transfected with podoplanin-GFP construct were incubated with the soluble cECD-rFc and rFc-cECD secreted from transfected 293 T cells. As shown in Fig. 3a, cECD-rFc could significantly bind to CHO cells with the expression of podoplanin and was widely colocalized with podoplanin. In contrast, there was a very weak binding of cECD-rFc to mock-transfected CHO cells, suggesting that the cECD-rFc protein is functionally active and has a specific binding with podoplanin. Similar results were observed in HeLa cells transfected with podoplanin-GFP construct (Fig. 3a). However, the soluble rFc-cECD did not significantly bind to CHO-K1 cells and HeLa cells transfected with podoplanin-GFP construct, suggesting that the rFc-cECD protein does not have a proper conformation and not bind podoplanin on cells (Fig. 3b). Our observation was further confirmed by a His-tag pull-down assay. HEK293T cells transfected with or without podoplanin construct were collected and lysed, followed by the incubation with Ni-NTA magnetic agarose beads. Then,

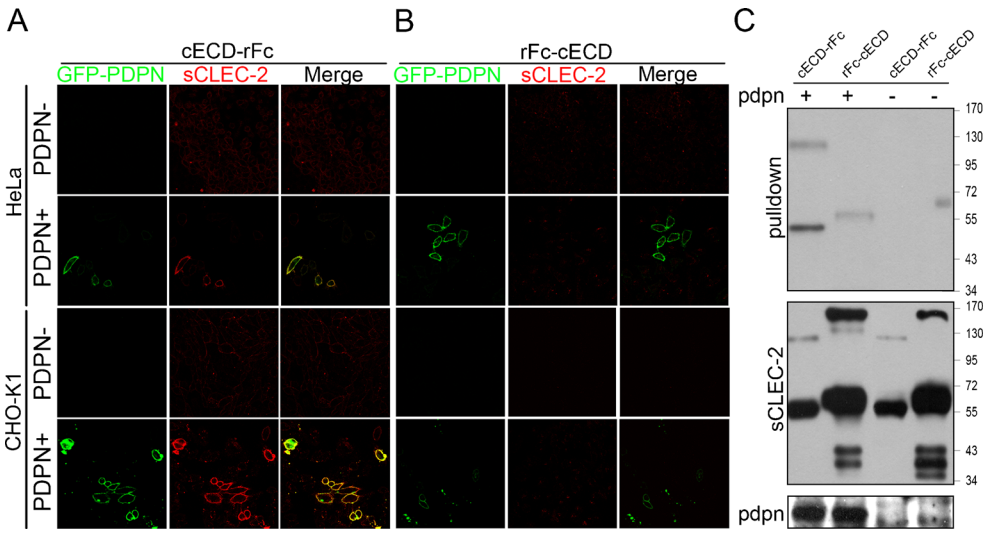

Fig. 3 Functional analysis of the recombinant CLEC-2. **a**, **b** HeLa cells and CHO-K1 cells transfected with pEGFP-PDPN were fixed and incubated with cECD-rFc (**a**) or rFc-cECD (**b**) and stained with Alexa Flour 549-conjugated goat anti-rabbit IgG antibody followed by confocal microscopy analysis. **c** HEK293T cells transfected with pcDNA3.1/Myc-His (+)-PDPN were lysed and incubated with recombinant CLEC-2 and Ni-NTA magnetic agarose beads. The coimmunoprecipitated complex was subjected to western blot analysis with goat anti-rabbit IgG antibody

the podoplanin-bound beads were incubated with an equal amount of rFc-cECD or cECD-rFc protein from transfected 293 T cells. As shown in Fig. 3c, podoplanin was able to bind both forms of low and high migration of cECD-rFc protein, suggesting that the glycosylation modification of cECD does not interfere with the binding of CLEC-2 with podoplanin. However, podoplanin only weakly bound the form of low migration of rFc-cECD protein, which confirms that the cECD region in this protein might not have a proper conformation and does not interact with podoplanin. In fact, it is likewise true for cECD-mFc and mFc-cECD (data not shown). Therefore, the pattern of cECD-Fc fusion order produces the functionally active recombinant proteins.

The pattern of ECD-Fc fusion order is a suitable pattern for other Group II transmembrane proteins

To determine whether our observation above is universal for other Group II transmembrane proteins, we used the receptors LOX-1 and Dectin-1 to address this issue. The constructs of the ECD of LOX-1 with rFc at C-terminus (lECD-rFc) or at N-terminus (rFc-lECD) were transfected into HEK293T cells, respectively, and the conditioned culture medium was collected and concentrated. The expression level was detected by western blot. As shown in Fig. 4a, two soluble forms of LOX-1 could be detected. Compared with the lECD-rFc protein, the rFc-lECD protein had a different conformation, resulting in different glycosyl-modification. In fact, similar results from the ECD of Dectin-1 with rFc at C-terminus (dECD-rFc) or at N-terminus (rFc-dECD) were observed, which further suggests that the Fc moiety at N-terminus of recombinant proteins plays a role in the protein folding (Fig. 4a). To determine the binding profile of the lECD-rFc and rFc-lECD proteins with heat shock protein 60 (Hsp60), CHO-K1 cells were transfected with HASPB-N18-Hsp60 construct to express Hsp60 on cells and were incubated with soluble LOX-1 followed by confocal microscopy analysis. As shown in Fig. 4b, the lECD-rFc protein, instead of the rFc-lECD protein, was able to co-localize with Hsp60 on cells, suggesting that the lECD-rFc protein, instead of the rFc-lECD protein, has a proper conformation to bind Hsp60 on cells. Furthermore, the flow cytometric analysis revealed that the dECD-rFc protein, but not the rFc-dECD protein, was capable to bind the exposed β(1-3) linked glucans on yeast cells (Fig. 4c), which further confirms that recombinant proteins with a C-terminal Fc moiety, but not N-terminal one, have the

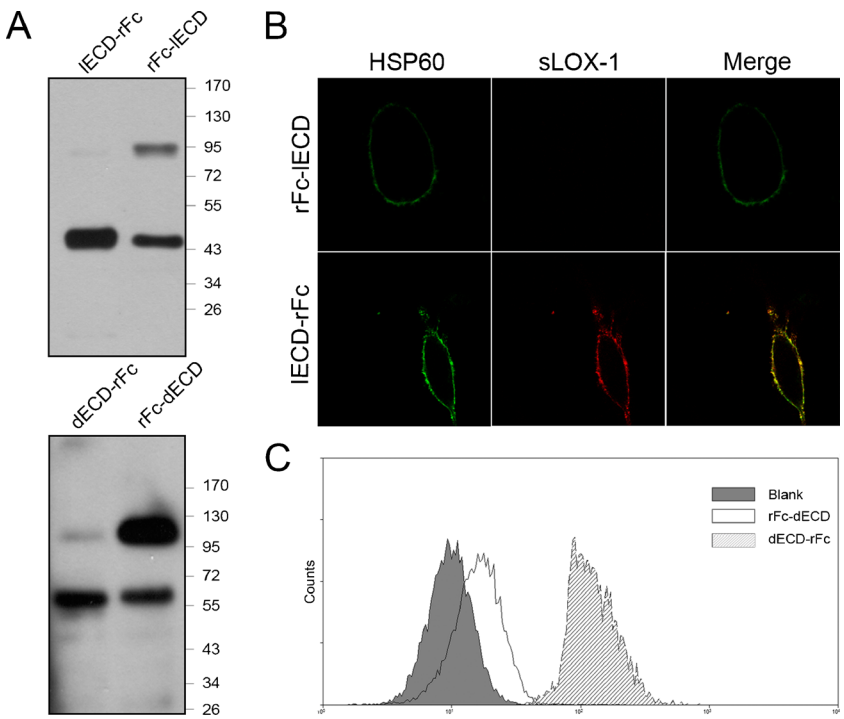

Fig. 4 The expression and binding analysis of recombinant LOX-1 and Dectin-1 **a** The culture medium of 293 T cells transfected with the indicated constructs was collected and concentrated under the same condition, and then subjected to western blotting with the HRP-conjugated antibody against rabbit Fc. lECD-rFc, the ECD of LOX-1 fused with the signal peptide of IL-2 at N-terminus and the rabbit Fc fragment at C-terminus; rFc-lECD, the rabbit Fc fragment fused with the signal peptide of IL-2 at N-terminus and the ECD of LOX-1 at C-terminus; dECD-rFc, the ECD of Dectin-1 fused with the signal peptide of IL-2 at N-terminus and the rabbit Fc fragment at C-terminus; rFc-dECD, the rabbit Fc fragment fused with the signal peptide of IL-2 at N-terminus and the ECD of Dectin-1 at C-terminus. **b** CHO-K1 cells transfected with HASPB-N18-Hsp60 construct were fixed and incubated with recombinant LOX-1, followed by staining with anti-Myc antibody and confocal microscopy analysis. **c** *Saccharomyces cerevisiae* AH109 yeast cells were boiled for 5 min and incubated with recombinant Dectin-1 at 4 °C followed by flow cytometric analysis

proper conformation. Therefore, the pattern of ECD-Fc fusion order might be suitable for generating recombinant ECDs of group II transmembrane proteins with the fusion of Fc moiety.

Discussion

In this study, our data showed that the glycosylation of recombinant cECD-Fc protein was different from that of recombinant Fc-ECD protein. N-linked glycosylation is the most common type of glycosidic bond and is important for the folding of some eukaryotic proteins and for cell-cell, cell-extracellular matrix interactions [4, 15]. Generally, N-linked glycans attach to the nitrogen of asparagine or arginine side-chains in the NXS/T sequence [16]. However, not all potential glycosylation sites are glycosylated, and not all glycosylated sites share the same glycosylation pattern that obviously affects the function and activity of the target proteins. The variability of the N-linked glycosylation depends on the culture condition, the location of the potential glycosylation sites, as well as the expression system [17]. In our study, the patterns of the Fc-cECD and cECD-Fc fusion order represented significantly different expression and function profiles of cECD, notwithstanding the same NXS/T motif that they shared. Though mFc and rFc had different potential glycosylation sites, they possessed a similar glycosylation profile. Therefore, we speculated that the altered glycosylations took place in the cECD region, which could be verified by the binding of receptor and its ligand. The fusion pattern of Fc and cECD might alter the structural conformation, which promoted the generation of the novel glycosylation modification. The change of structural conformation was also confirmed in different cell lines. Furthermore, the ECDs of LOX-1 and Dectin-1 with Fc moiety at N-terminus did affect their glycosylation modifications, which led to the decrease of the binding ability of LOX-1 and Dectin-1 with their ligands. These results suggest that the Fc moiety at the N-terminus of recombinant Group II transmembrane proteins indeed alters their structural conformation.

In this study, our results showed that the cECD-Fc fusion protein was functionally active while the Fc-cECD fusion protein was inactive. This could be demonstrated by the binding of CLEC-2 with its ligand podoplanin on cells. In fact, the same functional deviation also was observed in ECDs of other group II transmembrane proteins with the fusion of Fc moiety, such as LOX-1 and Dectin-1, which implies that it is a better usage of the pattern of ECD-Fc fusion order to express recombinant ECDs of Group II transmembrane proteins. It is unknown whether the glycosylation interferes with the binding of CLEC-2 to podoplanin. In fact, the glycosylation site of CLEC-2 is far away from the binding site of CLEC-2 with podoplanin and the glycosylation of CLEC-2 was reported not to be involved in the binding of CLEC-2 with podoplanin [18]. Therefore, the glycosylation of CLEC-2 seems not to interfere with the binding ability of CLEC-2 with podoplanin. Previous reports showed that the receptor domain of Fc-fusion protein influences the structural environment of the FcRn binding region but not of the FcgRI binding region of the Fc domain [19]. It is possible that the pattern of Fc-cECD fusion order impairs the native conformation of cECD, which leads to the abolishment of the binding ability of CLEC-2 with podoplanin.

We used the mouse IL-2 signal peptide to deliver the cargo to extracellular space. Our results indicate that the secretion of the mFc-fused protein is more efficient than that of rFc-fused one. The secretion efficiency might be regulated by the amino acid sequence of the cargo as well as the conformation. Since mFc and rFc have similar conformations, it is impossible that the secretion of cargo is regulated by the conformation of Fc moiety. The alignment of the 30 amino acids at the N-terminus of mFc and rFc moiety reveals a low similarity (10 %), which implies that the secretion of cargo might be modulated by the amino acid sequence after the signal peptide. On the other hand, the rFc-fused proteins are more sensitive for detection than mFc-fused ones. Although previous reports showed that antibodies derived from different species have different affinity against antigens, the antibody against mFc or rFc is derived from goat, which obviates the effect from species-derived bias. It is possible that rabbit Fc moiety is a more potential antigen than mouse Fc moiety, which results in the efficient antibodies against rFc. Therefore, it is better to use mFc-fused protein to purify the potential ligands, while it is better to use rFc-fused protein to detect potential ligands on cells.

Altogether, our study provides a favorable fusion pattern of Fc moiety with the ECDs of group II transmembrane proteins and simultaneously presents an alternative selection of mFc and rFc for different aims.

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