Europe PMC Funders Group
Author Manuscript
Biochem J. Author manuscript; available in PMC 2009 September 23.

Published in final edited form as:
Biochem J. 2008 April 1; 411(1): 133–140. doi:10.1042/BJ20071216.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Renal cells activate the platelet receptor CLEC-2 through podoplanin

Charita M. Christou[*,1], **Andrew C. Pearce**[†,1], **Aleksandra A. Watson**[*], **Anita R. Mistry**[*], **Alice Y. Pollitt**[†], **Angharad E. Fenton-May**[*], **Louise A. Johnson**[‡], **David G. Jackson**[‡], **Steve P. Watson**[†], and **Chris A. O'Callaghan**[*,2]

[*]Henry Wellcome Building for Molecular Physiology, University of Oxford, Roosevelt Drive, Oxford. OX3 7BN, UK

[†]Centre for Cardiovascular Sciences, Institute of Biomedical Research, The Medical School, University of Birmingham, Birmingham, B15 2TT, UK

[‡]The Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK

Abstract

We have recently shown that the C-type lectin-like receptor, CLEC-2, is expressed on platelets and that it mediates powerful platelet aggregation by the snake venom toxin, rhodocytin. In addition, we have provided indirect evidence for an endogenous ligand for CLEC-2 in renal cells expressing human immunodeficiency virus type 1 (HIV-1). This putative ligand facilitates transmission of HIV through its incorporation into the viral envelope and binding to CLEC-2 on platelets. The aim of this study was to identify the ligand on these cells which binds to CLEC-2 on platelets. Recombinant CLEC-2 exhibits specific binding to 293T cells in which the HIV can be grown. Further, 293T cells activate both platelets and CLEC-2-transfected DT-40 B cells. The transmembrane protein podoplanin was identified on 293T cells and demonstrated to mediate both binding of 293T cells to CLEC-2 and 293T cell activation of CLEC-2-transfected DT-40 B cells. Podoplanin is expressed on renal cells (podocytes). Further, a direct interaction between CLEC-2 and podoplanin was confirmed using surface plasmon resonance and was shown to be independent of glycosylation of CLEC-2. The interaction has an affinity of $24.5 \pm 3.7 \mu M$. The present study identifies podoplanin as a ligand for CLEC-2 on renal cells.

Keywords

Platelets; CLEC-2; podoplanin; C-type lectin-like receptor

Introduction

We have recently identified the C-type lectin-like receptor, CLEC-2, as a novel activating receptor that is expressed on the surface of platelets and megakaryocytes [1]. CLEC-2 can be activated by the snake venom toxin rhodocytin or by a specific antibody, triggering powerful platelet aggregation [1]. CLEC-2 signals through a single YXXL motif that is present in its cytoplasmic domain [2]. Crosslinking of CLEC-2 induces Src kinase-dependent tyrosine phosphorylation of the YXXL sequence, inducing activation of the tyrosine kinase Syk and initiation of a signalling pathway that culminates in activation of

[2]To whom correspondence should be addressed (chris.ocallaghan@ndm.ox.ac.uk).
[1]These authors contributed equally to this work.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

PLCγ2 [2]. The CLEC-2 signalling pathway is similar to, but distinct from, that used by receptors that signal through an immunoreceptor tyrosine-based activation motif (ITAM), such as the major signalling receptor for collagen on platelets, the GPVI-FcR γ-chain complex. These two signalling pathways differ in that activation of PLCγ2 by ITAM receptors has an absolute requirement for two YXXL motifs and the SLP-76/Blnk family of adapter proteins [3], whereas CLEC-2 has a single YXXL sequence and is only partially dependent on this family of adapter proteins [2].

CLEC-2 is a member of the C-type lectin-like family of receptors, but its putative carbohydrate recognition domain lacks the key structural features that confer binding to carbohydrates, suggesting that its endogenous ligand may be a protein. We have recently crystallized CLEC-2 and shown that it has a compact C-type lectin-like domain with a flexible loop on its surface [4]. A role for this loop in ligand binding is suggested by mutagenesis and surface plasmon resonance binding studies with rhodocytin [4].

CLEC-2 has recently been reported to facilitate capture of HIV-1 by CLEC-2-transfected cells and platelets [5]. Importantly, CLEC-2 does not bind to the HIV-1 envelope protein suggesting that CLEC-2 binds to a membrane protein derived from the HIV-producing 293T cells, that is captured by the virus during viral budding. Thus, 293T cells must express an endogenous ligand for CLEC-2. Infection of renal cells and production of infective virus from these cells is well documented in HIV infection and renal disease is an important complication of HIV infection [6, 7]. Therefore, this CLEC-2 interaction could be a significant mechanism in viral dissemination. The aim of the present study was to identify this ligand and to investigate its ability to bind and activate CLEC-2.

Experimental procedures

Recombinant proteins: cloning, production, purification and enzymatic biotinylation

For eukaryotic expression of glycosylated CLEC-2 protein, the extracellular domain of CLEC-2 was cloned into the plasmid pQCXIX (Clontech, CA) to encode a protein with an N-terminal 6-histidine tag followed by a BirA recognition sequence. The packaging cell line 293T-GP2 was transfected with the CLEC-2 expression construct, retrovirus harvested and used to infect 293T cells. The cell supernatant was centrifuged, filtered and protein purified by nickel affinity chromatography and size exclusion gel filtration using a Superdex 75 HR 26/60 column on an AKTA Purifier (GE Healthcare, Sweden).

For bacterial expression of unglycosylated protein, the extracellular domain of CLEC-2 was cloned into the plasmid pGMT7 to encode a protein with an N-terminal BirA recognition sequence. Recombinant CLEC-2 was expressed in *E. coli* strain BL21 DE3 pLysS as inclusion bodies as previously described [4, 8]. Briefly, inclusion bodies were isolated, washed in detergent, solubilized in guanidine, refolded in the presence of a redox couple and refolded protein purified by size exclusion chromatography.

Podoplanin was made as previously described [9]. Briefly, podoplanin was cloned into the plasmid pCDM7Ig, which was transiently transfected into 293T cells to express podoplanin fused at its C-terminus to the human IgG1 Fc region. The fusion protein was purified by protein A affinity chromatography and size exclusion chromatography.

BirA enzyme was produced and used to biotinylate proteins with BirA recognition tags as previously described [10]. A 4-fold molar excess of biotinylated protein was conjugated to phycoerythrin-labelled extravidin (Sigma, UK).

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Flow cytometry and competition experiments

Cells were washed in cold PBS 0.01% azide, blocked with 1% bovine serum albumin for 10 min on ice, washed again and incubated with fluorochrome-labelled multimeric CLEC-2 protein (3.5μM) for 1 hour. Cells were then washed 3 times, fixed in PBS 1% formaldehyde and analyzed on a FACS Canto machine (Becton-Dickinson, UK) and subsequently with FlowJo software (Tristar, CA, USA). For competition studies, cells were pre-incubated with free unlabelled CLEC-2 protein, NKG2D, rat monoclonal anti-human podoplanin antibody NZ1 (AngioBio, CA, USA) or isotype control for 1 hour before the addition of multimeric fluorochrome-labelled CLEC-2. The monoclonal anti-human podoplanin antibody 18H5 (Santa Cruz Biotechnology, CA, USA) was also used for flow cytometry. For platelet analysis, whole blood was isolated in tri-sodium citrate, directly labelled, then diluted prior to flow cytometry.

Functional studies in platelets and DT-40 transfected cell lines

Platelet rich plasma was isolated as previously described [11] and placed in a Born-lumiaggregometer with stirring at 1200 rpm at 37°C for 5 min before addition of 293T cells suspended in PBS. In some experiments, platelets were pre-incubated with the GPIIbIIIa antagonist, integrilin (9μM), or the Src kinase inhibitor, PD173952 (20μM; Pfizer, MI, USA), for 2 min prior to experimentation. Activation of CLEC-2 was monitored in DT-40 cells using an NFAT-luciferase reporter assay following transient transfection of CLEC-2 or the Y7F mutant of CLEC-2, as previously described [2]. The DT-40-transfected cells were incubated with 293T cells for 6 hours before measurement of luciferase activity. In competition experiments, 293T cells were pre-incubated with 20μg/ml rat IgG or rat anti-human podoplanin antibody NZ-1 on ice for 20 min prior to experimentation.

Metabolic labelling and Western blotting

Cells were resuspended in methionine-free medium and incubated for 1 hour. Cells were then incubated overnight in ^{35}S-labelled methionine (GE Healthcare, UK), then washed and lysed on ice in 0.5% NP40, 0.25M NaCl, 10mM Tris pH 7.5 with protease inhibitors. Lysates were pre-cleared with protein G agarose beads (Sigma, UK) and incubated with or without 100μg recombinant biotinylated CLEC-2 for 1 hour on ice with gentle agitation. Anti-polyhistidine antibody (Sigma, UK) was added for 1h and then protein G agarose was added and the mixture rotated at 4°C for 3 hours. Beads were washed 5 times, and proteins were eluted in Laemmli reducing sample buffer and boiled for 5min. Proteins were separated on 12% SDS-PAGE gels, which were dried and exposed to photographic film. Western blots were performed in an equivalent manner without metabolic labelling. Gels were blotted onto nitrocellulose membranes, which were blocked in 5% milk, washed and incubated with 1μg/ml NZ1, washed and incubated with anti-rat immunoglobulin-HRP antibody (Dako, UK).

Surface plasmon resonance binding studies

Surface plasmon resonance binding studies were conducted using a Biacore T100 machine (Biacore GE, Sweden) as described previously [12]. The biological activity of recombinant CLEC-2 and podoplanin were established by demonstrating binding of each fluorescence-labelled protein to cells transfected with the other protein. Proteins were attached to the carboxymethylated dextran-coated surface of CM5 biosensor chips, using amine coupling chemistry and experiments performed in 10mM Hepes pH 7.4, 150mM NaCl, 3mM EDTA and 0.005% polysorbate 20 surfactant. Non-specific interactions were controlled for by subtraction of the signal from a reference flow cell coated with an irrelevant protein. All CLEC-2 protein used in binding studies was monomeric. To avoid avidity effects with podoplanin which was expressed as a dimeric Fc-fusion protein, experiments were only

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

performed with podoplanin immobilized on the chip surface. Kd values were obtained by nonlinear curve fitting of the Langmuir isotherm to the data using the Levenberg-Marquardt algorithm as implemented in the program Origin (Microcal Software, MA, USA).

Results

CLEC-2 protein binds to a cell surface ligand on 293T cells

To confirm that 293T cells express a ligand for CLEC-2 and to provide a route to its identification, we expressed the extracellular domain of CLEC-2 in eukaryotic cells as a recombinant protein fused at its N-terminus to a BirA recognition sequence [10]. This allowed site-specific enzymatic biotinylation at the membrane insertion point of the protein. Biotinylated protein was tethered to a fluorochrome-labelled avidin derivative with four biotin-binding sites to create a species that is tetrameric with respect to CLEC-2 and in which CLEC-2 retains the orientation that it would have on the cell surface, with the C-terminus of the protein available for interaction with a ligand. Flow cytometry studies using this fluorochrome-labelled CLEC-2 demonstrated binding of CLEC-2 to 293T cells, consistent with expression of an endogenous ligand for CLEC-2 on these cells (Figure 1A). No binding was seen with the fluorochrome-labelled extravidin alone. To confirm that the interaction of multimeric CLEC-2 protein was specific, binding was carried out in the presence of free CLEC-2. Increasing concentrations of free unlabelled CLEC-2 inhibited binding of the fluorescence-labelled protein in a concentration-dependent manner, consistent with CLEC-2 exhibiting a specific mode of binding (Figures 1A and 1B). By contrast, incubation with a range of concentrations of a free irrelevant protein (NKG2D) did not inhibit binding of fluorescent CLEC-2 (Figure 1B).

To confirm that the binding seen was not artefactual due to the multimeric nature of the CLEC-2 used, the experiment was repeated using monomeric CLEC-2. When monomeric biotinylated CLEC-2 was bound to cells, it could be detected by the addition of fluorochrome-labelled extravidin (Figure 1C). However, the binding was slightly less as predicted by the loss of avidity effects in the primary binding of monomeric CLEC-2 to the cells.

A range of other cell types were also screened for binding to the CLEC-2 tetramer. High levels of specific binding (i.e. binding that could be competed out with unlabelled CLEC-2), comparable to that seen on 293T cells, were observed on SuSa cells, which are derived from testis. Intermediate levels of specific binding were observed on HT1080 and U2OS cells, which are derived from connective tissue and osteocytes respectively, and low level binding was observed on the erythroid/megakaryocyte progenitor cell line, K562, and on ovarian SKOV3 cells. In contrast, there was no specific binding on T cells (Jurkat), B cells (Raji, Daudi, CIR), natural killer cells (NK92), mammary epithelial cells (T47D), monocytes (U937 and HL60), platelets or primary endothelial cells (HUVEC). This data is summarised in Table 1 and representative binding as measured by flow cytometry is shown in Figure 1D.

Glycosylation of CLEC-2 is not required for ligand binding

CLEC-2 is able to interact with a range of different cell types of diverse origin, suggesting that the ligand is widely expressed. Native CLEC-2 in platelets is glycosylated [1] and recombinant CLEC-2 has a molecular mass of ~33kDa compared to the predicted molecular mass of ~26kDa (Figure 2A). Treatment of this protein with PNGaseF (peptide N-glycosidase F), which removes N-linked glycans, reduced the molecular weight to its predicted value (Figure 2A), confirming that recombinant CLEC-2 is glycosylated. To establish whether the binding of CLEC-2 to its ligand was dependent on carbohydrate groups on CLEC-2, unglycosylated CLEC-2 was produced in *E. coli*. This protein was

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

biotinylated and tethered to phycoerythrin-labelled extravidin and used for flow cytometry in the same manner as the CLEC-2 expressed from eukaryotic cells. Unglycosylated CLEC-2 binds to primary and cultured cells in a similar manner to glycosylated CLEC-2 as illustrated in Figure 2B and Table 1. Two other irrelevant C-type lectin-like molecules, made in a similar way in *E. coli* and refolded from inclusion bodies did not show any binding to the cells (data not shown). In addition, PNGaseF treated CLEC-2, which was made in eukaryotic cells, also bound to cells specifically, as demonstrated in Figure 2C. These observations demonstrate that sugar moieties on CLEC-2 are not necessary for CLEC-2 to bind to its ligand. Thus, recombinant CLEC-2 binds specifically to the surface of a number of cell types, including 293T cells, and this binding is independent of its glycosylation. These data strongly suggest that one, or possibly more than one, transmembrane protein functions as an endogenous ligand for CLEC-2.

293T cells induce platelet aggregation and activate CLEC-2 transfected DT-40 cells

The above results demonstrate the presence of a ligand for CLEC-2 on 293T cells and confirm our previous indirect evidence for this [5]. Studies were therefore undertaken to investigate whether this putative ligand is able to activate platelets. Figure 3A demonstrates that 293T cells stimulate powerful aggregation of platelets, with the magnitude of response being determined by the number of 293T cells used. Importantly, this response was inhibited by the GPIIbIIIa integrin antagonist, integrilin, demonstrating that it is true integrin-dependent aggregation rather than agglutination (Figure 3B). Furthermore, activation by 293T cells was inhibited in the presence of the Src kinase inhibitor, PD173952 (Figure 3B). This is consistent with the observation that CLEC-2 signals through a Src kinase dependent pathway [2]. Moreover, 293T cells also stimulated activation of FcR γ-chain deficient mouse platelets (data not shown) demonstrating that activation is independent of the platelet collagen receptor GPVI, the other major platelet activation receptor that induces aggregation via Src kinases.

To confirm that 293T cells are able to activate platelets via CLEC-2, we performed a series of studies on CLEC-2 transfected DT-40 cells, using an NFAT reporter assay [2]. Activation of CLEC-2 in this model cell line leads to induction of NFAT activity and expression of luciferase, which can be measured by luminometry. Strikingly, exposure to 293T cells increases NFAT activity in CLEC-2 transfected, but not mock-transfected, DT-40 cells (Figure 3C), confirming that they express a ligand that is able to activate the C-type lectin-like receptor. Moreover, DT-40 cells transfected with a mutant of CLEC-2 in which the cytoplasmic motif YXXL was changed to FXXL (Figure 3C) were not activated by 293T cells. A similar result has previously been reported for activation of CLEC-2 by rhodocytin in DT-40 cells [2]. Thus, these results confirm that 293T cells express a ligand for CLEC-2 that induces activation of the C-type lectin-like receptor through the same pathway as that used by the snake toxin, rhodocytin.

293T cells express the CLEC-2 ligand, podoplanin

To characterize the surface proteins in 293T cells that bind to CLEC-2, pull down experiments were performed with recombinant CLEC-2 on cells that had been metabolically labelled with ^{35}S-methionine. This approach identified several minor bands and a major band of 36 kDa that were not present in the control lane (Figure 4A). During the course of these studies, podoplanin, a 36 kDa protein that is expressed on certain tumours was shown to interact with CLEC-2 [13]. Podoplanin derives its name from podocytes which express it [14, 15], and which are kidney epithelial cells and therefore closely related to the human embryonic kidney 293T cells.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Using a specific antibody against podoplanin, we have demonstrated that recombinant CLEC-2 precipitates podoplanin from 293T cells and that this migrates in the same region as the 36kDa protein identified in the metabolic labelling studies (Figure 4B). Further, we have confirmed expression of podoplanin on 293T cells, with strong staining using an anti-podoplanin antibody (Figure 5A), comparable to the staining seen with recombinant CLEC-2 (Figure 1). Moreover, binding of the podoplanin antibody paralleled that of binding of recombinant CLEC-2 on a range of cell lines as shown in Figure 5B and Table 1. Thus, these observations strongly suggest that podoplanin is the ligand that confers binding of 293T and a number of other cell lines to CLEC-2. Direct confirmation that this was the case was obtained using a specific antibody to podoplanin, NZ-1, that has been shown to block binding of podoplanin-expressing cells to platelets [16]. As shown in Figure 5C, NZ-1 inhibited binding of recombinant CLEC-2 to 293T cells in a similar way to that observed with unlabelled CLEC-2 (Figure 1) confirming that binding was mediated by podoplanin. Moreover, NZ1 also blocked activation of CLEC-2 transfected DT-40 cells by 293T cells (Figure 3D), thereby confirming that 293T cells bind and activate CLEC-2 through podoplanin. In addition, recombinant podoplanin was able to activate CLEC-2 transfected DT-40 cells directly (Figure 5D), confirming that the interaction between podoplanin and CLEC-2 triggers signalling. Further, the signalling was dependent on the YXXL sequence of CLEC-2 confirming that it was mediated through the same mechanism as that used by rhodocytin (Figure 5D).

CLEC-2 and podoplanin interact directly with an affinity of 24.5 ± 3.7μM

Thus, not only does this work confirm that podoplanin is a specific ligand for CLEC-2, it also confirms the biological activity of the recombinant CLEC-2 and the recombinant podoplanin that was used in these studies. In order to confirm a direct interaction between CLEC-2 and podoplanin and determine the affinity of the interaction, we have used the two recombinant proteins in surface plasmon resonance binding studies (Figure 6). For these studies, podoplanin was tethered to a biosensor surface and interacted with different concentrations of recombinant CLEC-2. A progressively higher signal was seen with increasing concentrations of CLEC-2 protein. No interaction was seen with control proteins. The data were consistent with a single site binding model and demonstrate that the interaction between the two proteins is direct with an affinity of 24.5 ± 3.7μM.

Discussion

CLEC-2 is a newly characterized C-type lectin-like receptor, which has been shown to mediate platelet activation and aggregation upon binding to rhodocytin, a snake venom protein [1]. CLEC-2 has also been shown to enhance infectivity of HIV-1 produced in 293T cells [5]. This effect was not mediated by the viral envelope protein implying that a protein from the 293T cells was captured during viral budding and was responsible for the interaction of the virus with CLEC-2. These data suggested the presence of an endogenous, surface-bound ligand for CLEC-2 on 293T cells. We used recombinant CLEC-2 protein to confirm that 293T cells express a ligand for the receptor. This recombinant CLEC-2 was used to pull down potential ligand molecules from cell lysates and identified a candidate ligand of 36kDa that corresponds in size to podoplanin. We have shown that 293T and other CLEC-2 binding cells used in this study express podoplanin and that anti-podoplanin antibody inhibits both binding to and activation of CLEC-2 by 293T cells. A direct interaction between podoplanin and CLEC-2 was formally demonstrated by co-immunoprecipitation and surface plasmon resonance experiments, which determined the affinity to be 24.5μM.

These results are consistent with a report that certain cancer cells express podoplanin on their surface, which allows them to interact with CLEC-2 [13]. The study by Suzuki-Inoue

Europe PMC Funders Author Manuscripts

et al sought to identify a receptor for podoplanin and demonstrated that Src inhibitors blocked podoplanin-induced signalling through CLEC-2. Our study defines the signalling in CLEC-2 that is triggered by podoplanin and demonstrates that it is mediated through the YXXL motif in the cytoplasmic domain of CLEC-2. We have further demonstrated that glycosylation of CLEC-2 is not required for this interaction. There is good evidence that HIV infects renal cells, that these cells can produce infective HIV virions and that renal cells express podoplanin which may be incorporated into the budding virion [6, 14]. The present study has demonstrated that podoplanin binds directly to CLEC-2 with an affinity in the micromolar range, so inhibitors of this interaction may help prevent dissemination of HIV-1 by platelets.

Podoplanin was originally identified on a range of cells including airway epithelia, fibroblasts, keratinocytes, osteoblasts and renal tubular epithelial cells [17-20]. Podoplanin is also expressed at high levels on lymphatic endothelial cells [21] and on certain tumour cells, where it has been shown to activate platelets [16]. In the renal glomerulus, podoplanin helps maintain the structure of podocytes, required for efficient glomerular filtration [15]. Podocytes are not normally in contact with blood, but acute renal damage could expose them to blood, allowing interaction between CLEC-2 on platelets and podoplanin on podocytes. Physiologically, this would trigger platelet activation and formation of a haemostatic plug, thereby preventing further leakage into the collecting ducts, and possibly helping in renal repair through release of growth factors. However, this could also trigger intrarenal and especially intraglomerular thrombosis, which can be observed in renal diseases when there is renal epithelial damage, including during renal transplant rejection.

Inhibitors of the podoplanin-CLEC-2 interaction have potential use as novel anti-platelet agents. The significance of the interaction of podoplanin and CLEC-2 is unclear as the majority of cells that express podoplanin, with the exception of tumour cells undergoing metastasis, do not normally contact platelets. As with certain other C-type lectin-like receptors, it is possible that the function of CLEC-2 is primarily in defence, and specifically in this to prevent excessive blood loss and to respond to infection following contact with podoplanin. Further studies will provide information on the physiological and pathophysiological significance of the interaction of podoplanin and CLEC-2.

Acknowledgments

This work was supported by the Medical Research Council, the British Heart Foundation and the Wellcome Trust. We are grateful to Paul Harrison, Norma Masson, Neale Banerji, Alain Townsend, Hal Drakesmith, Lisa Schimanski, Emma Sweetland and Da Lin for helpful discussions.

Abbreviations used

ITAM immunoreceptor tyrosine-based activation motif

PNGaseF peptide N-glycosidase F

NFAT Nuclear Factor of activated T cells

References

1. Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y, Watson SP. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006; 107:542–549. [PubMed: 16174766]
2. Fuller GL, Williams JA, Tomlinson MG, Eble JA, Hanna SL, Pohlmann S, Suzuki-Inoue K, Ozaki Y, Watson SP, Pearce AC. The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN,

signal via a novel YXXL-dependent signaling cascade. J. Biol. Chem. 2007; 282:12397–12409. [PubMed: 17339324]

3. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. J. Thromb. Haemost. 2005; 3:1752–1762. [PubMed: 16102042]
4. Watson AA, Brown J, Harlos K, Eble JA, Walter TS, O'Callaghan CA. The crystal structure and mutational binding analysis of the extracellular domain of the platelet-activating receptor CLEC-2. J. Biol. Chem. 2007; 282:3165–3172. [PubMed: 17132623]
5. Chaipan C, Soilleux EJ, Simpson P, Hofmann H, Gramberg T, Marzi A, Geier M, Stewart EA, Eisemann J, Steinkasserer A, Suzuki-Inoue K, Fuller GL, Pearce AC, Watson SP, Hoxie JA, Baribaud F, Pohlmann S. DC-SIGN and CLEC-2 Mediate Human Immunodeficiency Virus Type 1 Capture by Platelets. J. Virol. 2006; 80:8951–8960. [PubMed: 16940507]
6. Marras D, Bruggeman LA, Gao F, Tanji N, Mansukhani MM, Cara A, Ross MD, Gusella GL, Benson G, D'Agati VD, Hahn BH, Klotman ME, Klotman PE. Replication and compartmentalization of HIV-1 in kidney epithelium of patients with HIV-associated nephropathy. Nat. Med. 2002; 8:522–526. [PubMed: 11984599]
7. Gupta SK, Mamlin BW, Johnson CS, Dollins MD, Topf JM, Dube MP. Prevalence of proteinuria and the development of chronic kidney disease in HIV-infected patients. Clin. Nephrol. 2004; 61:1–6. [PubMed: 14964451]
8. Watson AA, O'Callaghan CA. Crystallization and X-ray diffraction analysis of human CLEC-2. Acta. Crystallograph. Sect. F Struct. Biol. Cryst. Commun. 2005; 61:1094–1096. [PubMed: 16511244]
9. Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. Am. J. Pathol. 2005; 166:913–921. [PubMed: 15743802]
10. O'Callaghan CA, Byford MF, Wyer JR, Willcox BE, Jakobsen BK, McMichael AJ, Bell JI. BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation. Anal. Biochem. 1999; 266:9–15. [PubMed: 9887208]
11. Marshall SJ, Senis YA, Auger JM, Feil R, Hofmann F, Salmon G, Peterson JT, Burslem F, Watson SP. GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase. Blood. 2004; 103:2601–2609. [PubMed: 14684423]
12. O'Callaghan CA, Cerwenka A, Willcox BE, Lanier LL, Bjorkman PJ. Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. Immunity. 2001; 15:201–211. [PubMed: 11520456]
13. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Narimatsu H, Ozaki Y. Involvement of the snake toxin receptor CLEC-2 in podoplanin-mediated platelet activation by cancer cells. J. Biol. Chem. 2007; 282:25993–26001. [PubMed: 17616532]
14. Koop K, Eikmans M, Baelde HJ, Kawachi H, De Heer E, Paul LC, Bruijn JA. Expression of podocyte-associated molecules in acquired human kidney diseases. J. Am. Soc. Nephrol. 2003; 14:2063–2071. [PubMed: 12874460]
15. Matsui K, Breitender-Geleff S, Soleiman A, Kowalski H, Kerjaschki D. Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. Nephrol. Dial. Transplant. 1999; 14 Suppl 1:9–11. [PubMed: 10048437]
16. Kato Y, Kaneko MK, Kuno A, Uchiyama N, Amano K, Chiba Y, Hasegawa Y, Hirabayashi J, Narimatsu H, Mishima K, Osawa M. Inhibition of tumor cell-induced platelet aggregation using a novel anti-podoplanin antibody reacting with its platelet-aggregation-stimulating domain. Biochem. Biophys. Res. Commun. 2006; 349:1301–1307. [PubMed: 16979138]
17. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am. J. Pathol. 1997; 151:1141–1152. [PubMed: 9327748]
18. Dobbs LG, Williams MC, Gonzalez R. Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. Biochim. Biophys. Acta. 1988; 970:146–156. [PubMed: 3382696]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

19. Gandarillas A, Scholl FG, Benito N, Gamallo C, Quintanilla M. Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis. Mol. Carcinog. 1997; 20:10–18. [PubMed: 9328432]

20. Wetterwald A, Hoffstetter W, Cecchini MG, Lanske B, Wagner C, Fleisch H, Atkinson M. Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. Bone. 1996; 18:125–132. [PubMed: 8833206]

21. Breiteneder-Geleff S, Soleiman A, Horvat R, Amann G, Kowalski H, Kerjaschki D. [Podoplanin--a specific marker for lymphatic endothelium expressed in angiosarcoma]. Verh. Dtsch. Ges. Pathol. 1999; 83:270–275. [PubMed: 10714221]

Europe PMC Funders Author Manuscripts

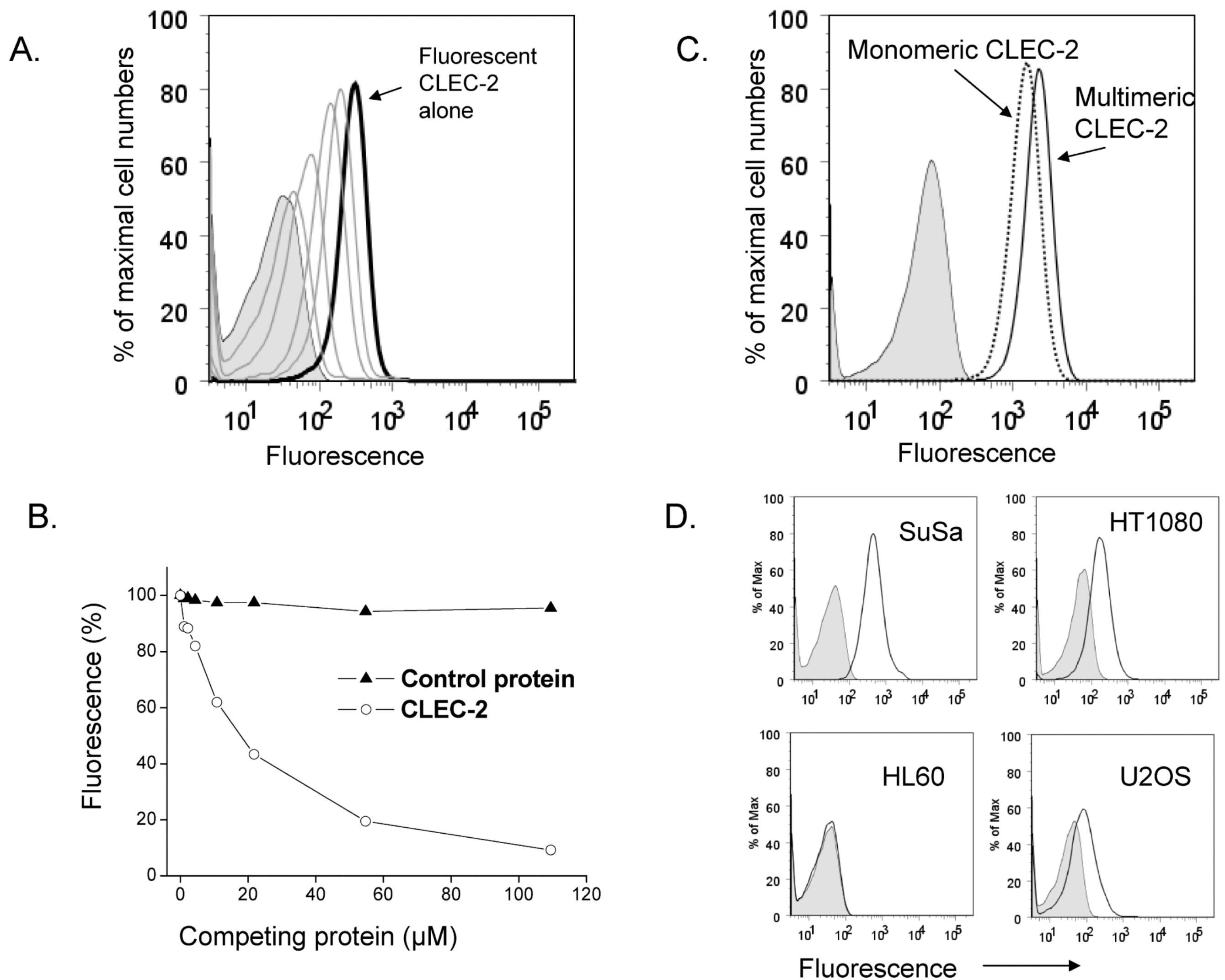

Figure 1. CLEC-2 binds specifically to 293T cells

A. Flow cytometry histograms of 293T cells following incubation with fluorescence-labelled CLEC-2. The thick black line indicates fluorescence of cells that have been incubated with CLEC-2 conjugated to extravidin-phycoerythrin. The shaded grey area represents background fluorescence in cells incubated with extravidin-phycoerythrin alone. Progressively higher concentrations of free unlabelled CLEC-2 competed off the fluorescence labelled-CLEC-2 as indicated by the unshaded pale grey histograms.

B. The graph illustrates the specific concentration-dependent reduction in binding of fluorescence-labelled CLEC-2 that occurs when free monomeric CLEC-2 is present, but not when an irrelevant protein (NKG2D - a different C-type lectin-like receptor) is present. This is consistent with specific binding of fluorescence-labelled CLEC-2 with a ligand on 293T cells.

C. Flow cytometry histograms of 293T cells after incubation with multimeric fluorescence-labelled CLEC-2 (black line) or monomeric biotinylated CLEC-2 (dotted line) detected after incubation with extravidin-phycoerythrin. Grey filled histogram represents background fluorescence with extravidin-phycoerythrin alone. The concentration of both monomeric and tetrameric CLEC-2 used was 3.5μM.

Europe PMC Funders Author Manuscripts

D. Representative flow cytometry histograms demonstrating different levels of binding with fluorescence-labelled CLEC-2 to the cell types indicated. The shaded areas represent background fluorescence with the extravidin-phycoerythrin alone.

The results in parts A - C are representative of between 3-5 experiments.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

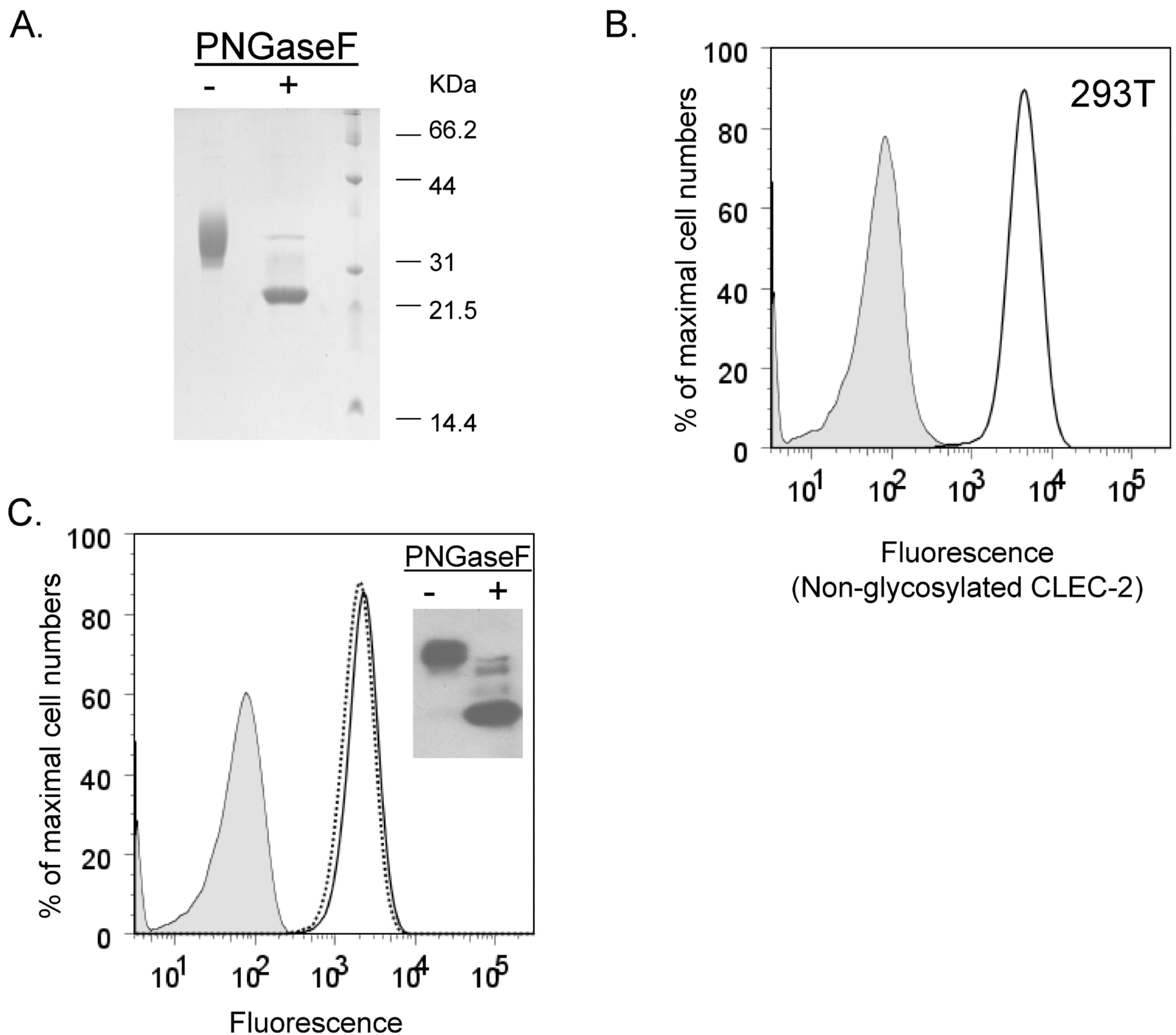

Figure 2. Glycosylation of CLEC-2 is not required for ligand binding

A. SDS-PAGE analysis of eukaryotically expressed CLEC-2 before and after treatment with PNGaseF, demonstrating that deglycosylation substantially reduces the size of CLEC-2.

B. Flow cytometry of 293T cells stained with fluorescence-labelled unglycosylated CLEC-2 produced in *E. coli* (black line). The shaded grey area represents the background fluorescence in cells incubated with extravidin-phycoerythrin alone. The results are representative of 3 experiments.

C. Flow cytometry of 293T cells stained with fluorescent glycosylated CLEC-2 (black line) and with deglycosylated CLEC-2 (dotted line) after digestion with PNGaseF. Shaded area represents background fluorescence. Inset is a Western blot of glycosylated CLEC-2 used for the staining before and after treatment with PNGaseF, demonstrating deglycosylation.

Europe PMC Funders Author Manuscripts

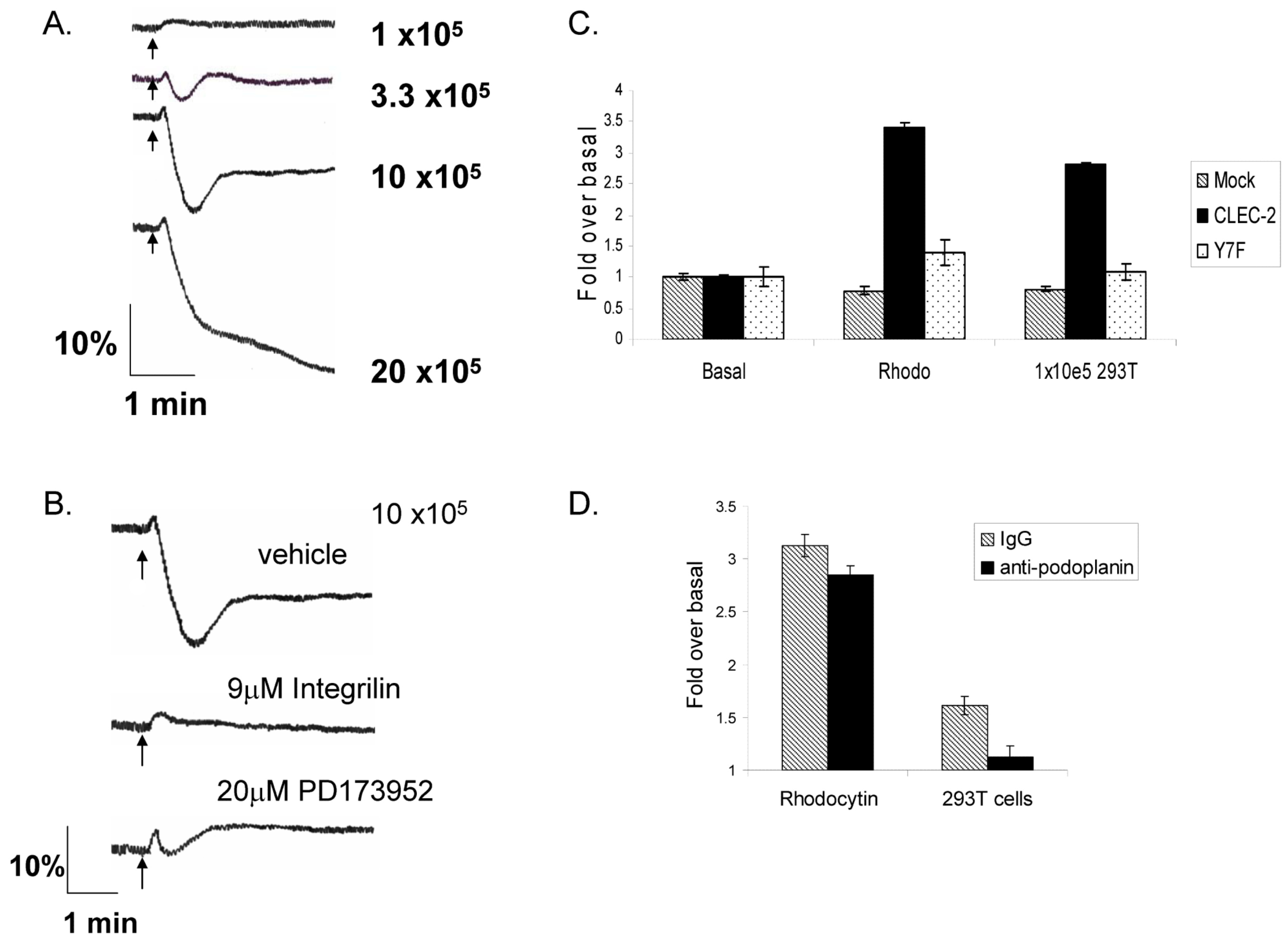

Figure 3. 293T cells induce platelet aggregation and activate CLEC-2 transfected DT-40 cells

A. Freshly isolated platelets suspended in platelet rich plasma were placed in a lumiaggregometer for 5 minutes at 37°C before addition of increasing numbers of 293T cells as indicated by the number on the right hand side. The time and % aggregation are indicated in the left hand corner, with 100% aggregation representing the difference in optical density between the platelet suspension and water.

B. Platelets suspended as above were pre-incubated with integrilin, a GPIIbIIIa antagonist, or PD173952, a Src kinase inhibitor, for 2 minutes prior to addition of 293T cells. The time and % aggregation are indicated in the left hand corner.

C. NFAT-luciferase activity was measured in DT-40 cells transiently transfected with CLEC-2 or with the Y7F CLEC-2 mutant. Transfected cells were incubated with rhodocytin or 293T cells for 6 hours before measuring luciferase activity.

D. NFAT-luciferase activity was measured in DT-40 cells transiently transfected with CLEC-2. Transfected cells were incubated with rhodocytin or 293T cells in the presence of the anti-podoplanin antibody, NZ1 (20ug/ml) or rat IgG2a (20ug/ml), before measuring luciferase activity.

The results in parts A - D are representative of between 3 - 5 experiments. In parts C and D, the error bars represent the standard error of the replicates within the experiment illustrated.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

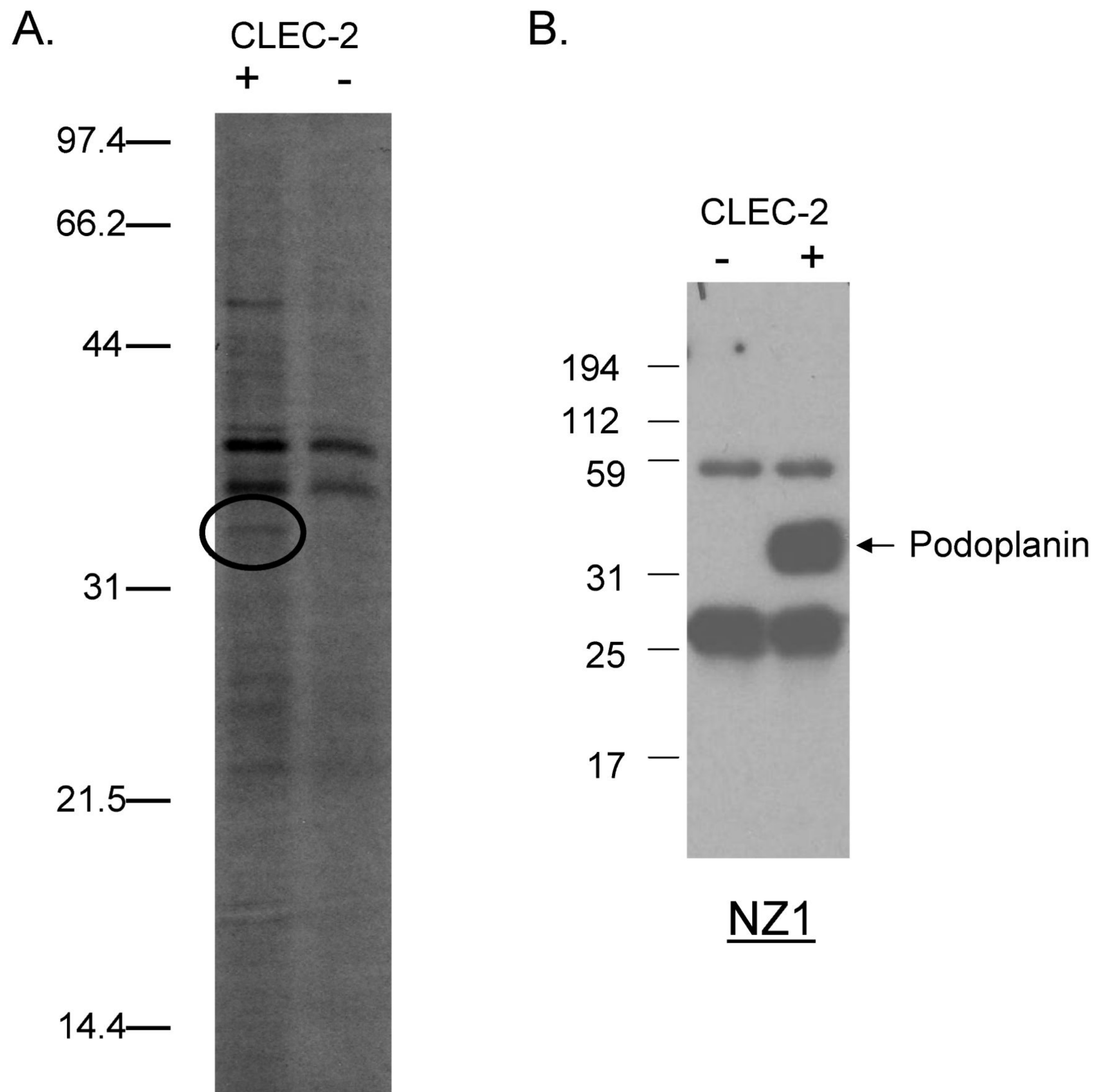

Figure 4. Co-precipitation demonstrates a podoplanin-CLEC-2 interaction

A: Co-precipitation: Metabolic labelling. Cells were labelled with ^{35}S-methionine overnight and then cell lysates precipitated with recombinant CLEC-2 using anti-polyhistidine antibody and protein G agarose beads. Bound protein was eluted, separated by SDS-PAGE and visualised by autoradiography.

B: Co-precipitation: Western blotting. Cells were lysed and incubated with or without recombinant eukaryotic CLEC-2, and then precipitated with anti-polyhistidine antibody and protein G agarose beads. Proteins were separated on SDS-PAGE, blotted onto a nitrocellulose membrane and detected with the anti-podoplanin antibody NZ1. The upper and lower bands represent the heavy and light chains of the anti-polyhistidine antibody respectively.

The results in parts A-B are representative of 3-5 experiments.

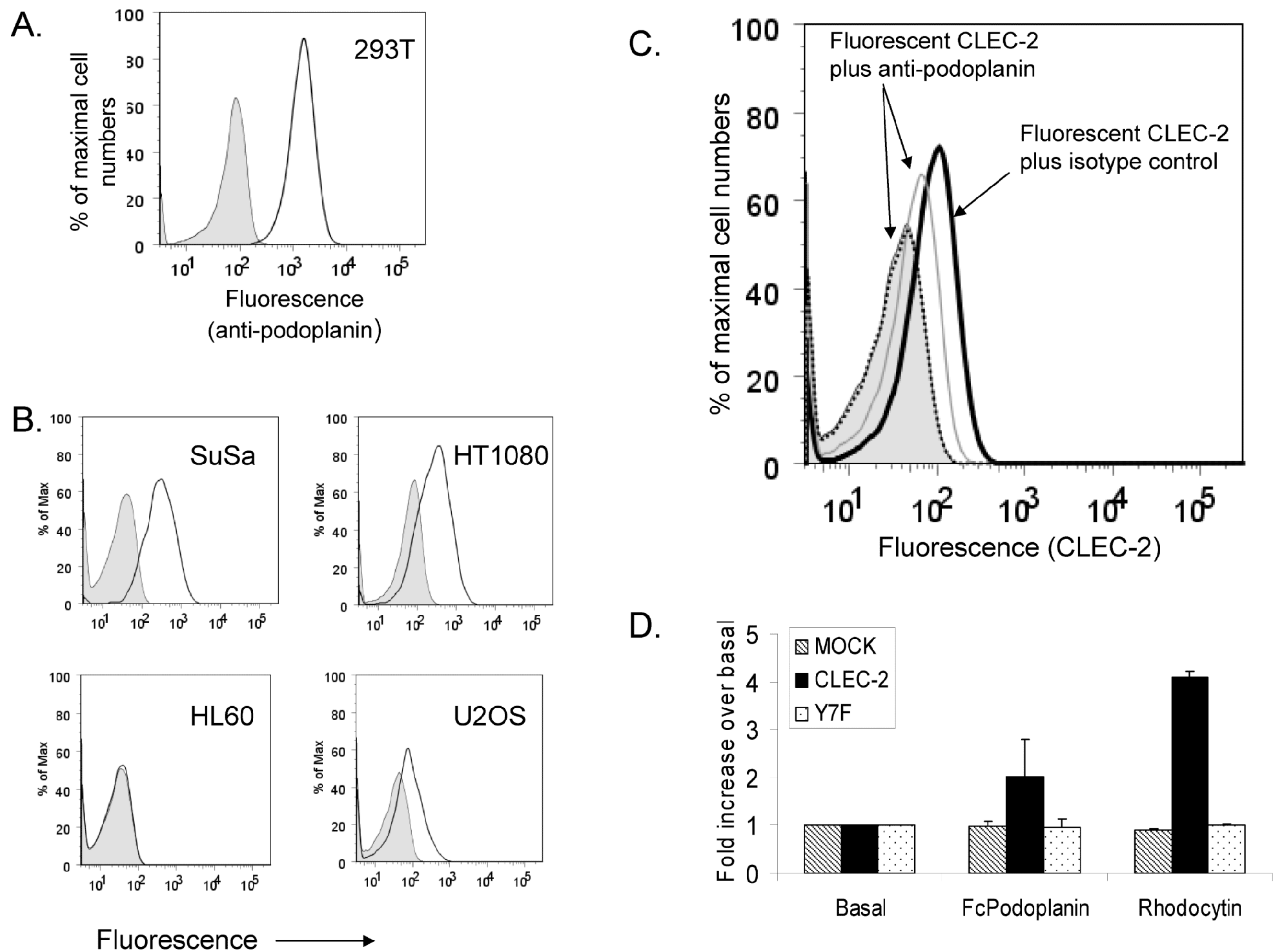

Figure 5. Podoplanin is a ligand for CLEC-2 on 293T cells

A. Flow cytometry histogram of 293T cells stained with anti-podoplanin antibody (black line). The shaded area represents an isotype control.

B. Representative flow cytometry histograms demonstrating different levels of staining with anti-podoplanin antibody. The shaded areas represent isotype controls.

C. Inhibition of CLEC-2 binding to 293T cells by anti-podoplanin antibody. Increasing concentrations of anti-podoplanin antibody (grey line, dotted line) progressively reduce binding to the level of background fluorescence (shaded area).

D. NFAT-luciferase activity was measured in DT-40 cells transiently transfected with CLEC-2 or with Y7F-CLEC-2. Transfected cells were incubated with recombinant podoplanin (Fc fusion) or with rhodocytin, demonstrating that recombinant podoplanin stimulates DT-40 cells via interaction with CLEC-2.

The results in parts A - C are representative of between 3 - 5 experiments, and those in part D of 2 experiments.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

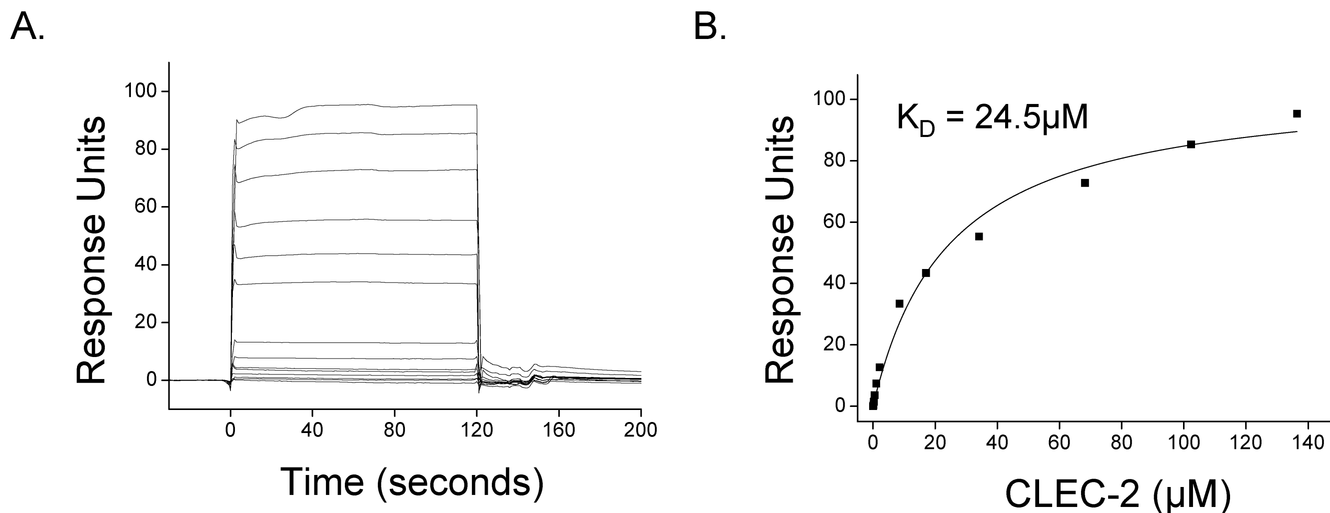

Figure 6. CLEC-2 interacts directly with podoplanin

A. Sensorgrams from typical equilibrium-based binding experiments after subtraction of the background response from a control surface. Different concentrations of CLEC-2 were injected over surfaces coupled with podoplanin, producing a concentration-dependent signal. B. Plot of the equilibrium binding response from the sensorgrams as a function of CLEC-2 concentration. The curve is the best fit to the experimental data and is consistent with an affinity of 24.5 ± 3.7μM.

The results in parts A and B are representative of 3 experiments.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Table 1
Summary of CLEC-2 and anti-podoplanin antibody binding to cells

Cell Line	Glycosylated fluorescent CLEC-2	Non-glycosylated fluorescent CLEC-2	Anti-Podoplanin antibody
Daudi	-	-	-
U937	-	-	-
HL60	-	-	-
Jurkat	-	-	-
K562	+	+	+
293T	+++	+++	+++
SuSa	+++	ND	+++
T47D	-	ND	-
HT1080	++	ND	++
SKOV3	+	+	+
U2OS	++	++	++
Platelets	-	-	-
HUVEC	-	-	-

A summary of the binding of the glycosylated and non-glycosylated forms of fluorescent CLEC-2 and of the anti-human podoplanin antibody to different cell types. The results are representative of 2-4 experiments.

[-] no expression

[+] low expression

[++] moderate expression

[+++] high expression, ND, not determined.