Three-dimensional interplay among the ligand-binding domains of the urokinase-plasminogen-activator-receptor-associated protein, Endo180

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Endo180, also known as the urokinase plasminogen activator receptor (uPAR)-associated protein (uPARAP), is one of the four members of the mannose receptor family, and is implicated in extracellular-matrix remodelling through its interactions with collagens, sugars and uPAR. The extracellular portion of Endo180 contains an amino-terminal cysteine-rich domain, a single fibronectin type II domain and eight C-type lectin-like domains. We have purified a soluble version of Endo180 and analysed it by single-particle electron microscopy to obtain a three-dimensional structure of the N-terminal part of the protein at a resolution of 17 Å and reveal, for the first time, the interactions between non-adjacent domains in the mannose receptor family. We show that for Endo180, the cysteine-rich domain contacts the second C-type lectin-like domain, thus providing structural insight into how modulation of its several ligand interactions may regulate Endo180 receptor function.

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INTRODUCTION

The mannose receptor family comprises four members, the mannose receptor, the M-type phospholipase A₂ receptor (PLA₂R), DEC205 and Endo180. These transmembrane glycoproteins share a common structural arrangement of an amino-terminal cysteine-rich domain followed by a fibronectin type-II domain (FNII), and eight (ten in the case of DEC205) C-type lectin-like domains (CTLDS; Fig. 1A). It is the presence of several CTLDS within a single polypeptide backbone that makes this subgroup unique within the C-type lectin superfamily. After a single transmembrane

domain is a short cytoplasmic domain, which directs the trafficking of these receptors between the plasma membrane and the endosomal apparatus. This recycling property means that all family members can mediate the uptake of extracellular ligands for intracellular delivery (East & Isacke, 2002).

There is no high-resolution data for Endo180 domains, but atomic structures exist from homologous proteins. The atomic structure of the cysteine-rich domain of the mannose receptor (Liu *et al.*, 2000) shows a β-trefoil structure with threefold symmetry, similar to the soybean trypsin inhibitor and the R-type carbohydrate-recognition domains of ricin. In the mannose receptor, this domain binds oligosaccharides that terminate in GalNAc-4-SO₄ (Fiete *et al.*, 1998). However, it has been shown that neither DEC205 nor Endo180 cysteine-rich domains bind sulphated sugars (Leteux *et al.*, 2000; East *et al.*, 2002). The FNII domain is the most highly conserved between family members and, for Endo180, shares 45% and 42% sequence identity with the FN¹F2 and ²F2 gelatin/collagen-binding domains, respectively (East & Isacke, 2002). The solution structure of the FN¹F2 domain shows two double-stranded, anti-parallel sheets, which are orientated to form a solvent-exposed hydrophobic surface (Pickford *et al.*, 1997). It has been shown that both PLA₂R and Endo180 function as collagen-binding/internalization receptors (Ancian *et al.*, 1995; Engelholm *et al.*, 2003; Wienke *et al.*, 2003). A CTLD consists of two α-helices and two anti-parallel β-sheets (Weis *et al.*, 1992) and, typically, these domains mediate Ca²⁺-dependent sugar binding. However, in the mannose receptor family, in most of the CTLDS the crucial residues required for coordination of Ca²⁺ ions and sugar residues are not conserved. Both Endo180 and the mannose receptor are functional C-type lectins, but this activity is restricted to a single CTLD in each receptor, Endo180 CTLD2 and mannose receptor CTLD4 (Taylor *et al.*, 1992; East *et al.*, 2002).

Endo180 can also associate on the cell surface in a trimolecular complex with urokinase-type plasminogen activator (uPA) and the uPA receptor (uPAR; Behrendt *et al.*, 2000) hence its alternative name, uPAR-associated protein (uPARAP). In addition to its proteolytic function, the uPA/uPAR system also drives intracellular events that control cell adhesion and migration. However, the

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Fig. 1 | Purification of the Endo180 extracellular domain. **(A)** Domain structure of Endo180. The arrangement of the extracellular domains reflects the model proposed for the mannose receptor (Napper *et al.*, 2001). **(B)** Purified sEndo180–HA–His₆ (a soluble Endo180 extracellular-domain construct that contains a carboxy-terminal haemagglutinin (HA) tag and a six-histidine (His₆) tag) was immunoblotted using the monoclonal antibody E1/183 (directed against the human Endo180 cysteine-rich domain) or the anti-HA monoclonal 3F10, or was silver stained. **(C)** Purified sEndo180–HA–His₆ protein was loaded onto a GlcNAc-sepharose column. The column was washed eight times with 1 ml of loading buffer (containing 25 mM Ca²⁺) and then eight times with 1 ml of elution buffer (containing 10 mM EDTA). Fractions were precipitated and analysed by immunoblotting with the anti-Endo180 monoclonal antibody A5/158. CTLD, C-type lectin-like domain; Cys, cysteine-rich domain; cyto, cytoplasmic domain; FNII, fibronectin type II domain.

lack of a uPAR cytoplasmic domain requires a co-transmembrane receptor to mediate these latter responses. Hence, it is of interest that Endo180 is highly upregulated in tumour endothelium (St Croix *et al.*, 2000).

An important unanswered question is whether there is any interaction of domains within the three-dimensional structure of these receptors that could provide a mechanism for ligand-binding regulation. Velocity sedimentation and hydrodynamic modelling of soluble mannose-receptor constructs predict a monomeric, extended, relatively rigid conformation (Napper *et al.*, 2001; see Fig. 1A). To extend these studies for Endo180, and in particular to determine whether there are interactions between non-adjacent domains, we have used single-particle electron microscopy (EM), which allows three-dimensional structures of macromolecules in close-to-native conformations to be obtained.

RESULTS AND DISCUSSION

Endo180 purification

A soluble Endo180 extracellular-domain construct that contains a carboxy-terminal haemagglutinin (HA) tag and a six-histidine tag (sEndo180–HA–His₆) was expressed in COS-1 cells and purified on a nickel column. The resulting material was resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% gel, and this was subjected to silver staining and to immunoblotting with the monoclonal antibodies E1/183 (which binds to the Endo180 cysteine-rich domain) and 3F10 (which is directed against the C-terminal HA tag). A single band was seen (Fig. 1B), showing that the sEndo180–HA–His₆ protein had not been degraded during purification. Endogenous Endo180 is comprised of a 150-kDa protein core, which is modified by 30 kDa of *N*-linked oligosaccharides (Sheikh *et al.*, 2000). The observed 180-kDa size of the sEndo180–HA–His₆ protein indicates that it has been glycosylated in the COS-1 cells, although we cannot exclude a different glycosylation profile to that of the endogenous

protein. To confirm that the purification procedure did not disrupt ligand binding, the sEndo180–HA–His₆ protein was tested for its ability to bind to a GlcNAc-sepharose column. As previously described for intact Endo180 isolated from cells (East *et al.*, 2002), sEndo180–HA–His₆ bound to the GlcNAc column, and was eluted in the presence of EDTA (Fig. 1C). Similarly, it has been shown that sEndo180–HA–His₆ retains collagen-binding activity (Wienke *et al.*, 2003).

Electron microscopy

To observe the purified sample under the electron microscope, sEndo180–HA–His₆ was applied to a carbon-coated grid (Fig. 2A). After scanning of the electron micrographs, individual images were extracted and the contrast reverted (Fig. 2B). The images were then classified into groups that corresponded to similar projection angles using an iterative refinement (Ludtke *et al.*, 1999). Images within each class were then aligned to obtain averages with a high signal-to-noise ratio (Fig. 2C; rows 1b, 2b and 3b show a collection of averages from some of these classes). In the last refinement step, all these classes were used to generate the final volume. When this volume is projected, the projections (Fig. 2C, rows 1a, 2a and 3a) and averages (Fig. 2C, rows 1b, 2b and 3b) correlate well with each other. Resolution was estimated by Fourier shell correlation (Fig. 2D), and the cut-off of a 0.5 correlation coefficient was chosen to filter the final volume at a resolution of 17 Å. When the angular distribution of the Endo180 images that contributed to build this volume was analysed (Fig. 2E), we found a wide range of orientations, although it was clear that some classes were poorly represented. This suggested some orientation preference when the protein was bound to the grid. The protein shape can probably preclude some of the binding orientations, because the most highly populated classes (see averages at the top of Fig. 2E) correspond to side views of the volume (see below).

Three-dimensional reconstruction of Endo180

The Endo180 volume (Fig. 3) consisted of several differentiated domains. We assigned each of these to one segment of the Endo180 molecule by taking into account the arrangement of domains in its sequence and their published atomic structures from homologous protein, which allows us to calculate the overall shape and dimensions for each individual domain. This assignment was later confirmed by docking of the corresponding atomic structures (see below). From this analysis, we first located the clearly triangular

cysteine-rich domain, which corresponds to the most N-terminal part of the protein. Both the tip and one side of this triangle contact the neighbouring FNII domain. The next recognizable domain is a globular structure that corresponds to CTLD1, the general shape of which agrees with the atomic structure of the homologous CTLD from man-nose-binding protein A (MBPA; Weis *et al.*, 1992). MBPA and the mannose receptor CTLD4 (Feinberg *et al.*, 2000) have their N and C termini at the same side, and the Endo180 CTLD1 volume seen here shows a similar arrangement. Our structure clearly shows that CTLD1

Fig. 2 | Electron microscopy of purified Endo180. **(A)** Micrograph. Particles of sEndo180-HA-His₆ (a soluble Endo180 extracellular-domain construct that contains a carboxy-terminal haemagglutinin (HA) tag and a six-histidine (His₆) tag) are visible in negative contrast. **(B)** Extracted images after contrast inversion. **(C)** Classes obtained from the input images. Rows 1b, 2b and 3b are averages of the images of a given class. Images in rows 1a, 2a and 3a are the corresponding projections of the final volume. **(D)** Resolution estimation by Fourier shell correlation. **(E)** Angular coverage of Endo180 particles. The number of particles (y axis) for each class average (x axis) are shown. The average images of four of the most highly populated classes are shown (insets). The numbers at the bottom right-hand corners of the images indicate the class number.

Fig. 3 | Endo180 volume. (A–C) Views of the reconstructed volume for sEndo180–HA–His₆ (a soluble Endo180 extracellular-domain construct that contains a carboxy-terminal haemagglutinin (HA) tag and a six-histidine (His₆) tag). CTLD, C-type lectin-like domain; Cys, cysteine-rich domain; FNII, fibronectin type II domain. Scale bar, 32 Å.

and CTLD2 are in close proximity, as predicted for the mannose receptor (Napper *et al.*, 2001; see model in Fig. 1A). CTLD2 has two remarkable features: first, it contacts the triangular cysteine-rich domain, which may be important for modifying the otherwise extended conformation of the receptor. Second, CTLD2 has rotated in relation to CTLD1 (see side views of the volume in Fig. 3). After CTLD2, only a small extension, which probably represents part of the CTLD2–CTLD3 linker region, was reconstructed, showing that we were not visualizing the whole Endo180 construct. For EM, a size limit exists for observing single molecules with sufficient detail to obtain structural information. In theory, images from proteins of ~10 kDa or above should contain enough information to be aligned into a three-dimensional volume (Henderson, 1995). In practical terms, small molecules usually provide low contrast, so that this size limit would probably be higher. The use of a staining agent to increase contrast should therefore help to solve low-molecular-weight proteins by EM. Microscopists are trying to reach these size limits, and several small proteins have recently been reconstructed by EM (for example, the negatively stained, ~90 kDa protein, prefoldin; Martin-Benito *et al.*, 2002) and by cryomicroscopy (for example, the ~200 kDa spliceosomal U1 ribonucleoprotein (Stark *et al.*, 2001) and DPPIV/CD26 (Ludwig *et al.*, 2003)). We reconstructed only the Endo180 N terminus, which reflects the interactions of the first four domains to form a globular structure that can be visualized using an electron microscope. By contrast, if CTLDs 3–8 form an extended structure, as predicted for the mannose receptor (Napper *et al.*, 2001), then the rest of Endo180 would not be observed by EM.

To further verify this hypothesis, as well as the domain assignment, we examined a new construct, Endo180ΔCTLD3–8–Fc (East *et al.*, 2002), which only contained the cysteine-rich, FNII, CTLD1 and CTLD2 domains of Endo180, fused at their C terminus to an Fc tag to aid purification (Fig. 4A). The purified Endo180ΔCTLD3–8–Fc protein was resolved as a single band by SDS–PAGE, as determined by silver staining and by immunoblotting with monoclonal antibodies directed against the cysteine-rich domain and Fc tag (Fig. 4B). Moreover, this construct retained C-type lectin activity (East *et al.*, 2002) and collagen binding (data not shown), which is consistent with it containing the FNII and CTLD2 domains. Using an electron microscope, particles of two sizes were seen. The smaller individual images were less abundant, and they were collected (Fig. 4C) and used in a pattern-free refinement to obtain a three-dimensional structure (Fig. 4D). The volume had similar dimensions and overall

Fig. 4 | The Endo180ΔCTLD3–8–Fc construct. (A) The Endo180ΔCTLD3–8–Fc protein. (B) Left and middle panels show immunoblotting of purified Endo180ΔCTLD3–8–Fc protein with the anti-Endo180 monoclonal antibody E1/183 and an anti-human-Fc monoclonal antibody. Right panel shows a silver-stained SDS–polyacrylamide gel containing the purified material. Note that the size of the protein on the non-reducing SDS gel reflects Fc-mediated dimerization. (C) Images obtained for the construct using an electron microscope. (D) Views of the reconstructed volume. CTLD, C-type lectin-like domain; Cys, cysteine-rich domain; FNII, fibronectin type II domain.

domain distribution to the reconstructed Endo180 (plus a C-terminal Fc tag), and therefore confirms the three-dimensional arrangement for the N-terminal domains of this receptor. Larger particles, which were more abundant, and of roughly twice the size of those used in this reconstruction, were also selected (not shown). After a few rounds of refinement, it was apparent that these images probably represented dimerization of the protein through the Fc tail.

In apparent contrast with our observed conformation for the N-terminal domains of Endo180, Napper *et al.* (2001) have proposed that the related mannose receptor has a relatively rigid extended conformation. Two considerations can be made in this respect. First, Napper and colleagues used computer modelling to identify conformations consistent with their data. If, as we speculate, the folded conformation of Endo180 is restricted to the four most N-terminal domains within an overall extended conformation for the remaining part of the protein, such a variation might still be consistent with their data. Second, it is possible that the mannose receptor and Endo180 differ in the structural arrangements of their N-terminal domains. In Endo180, C-type lectin activity is mediated by CTLD2, whereas in the mannose receptor this activity resides in CTLD4. Consequently, the differences in their three-dimensional structures may reflect their differences in ligand binding.

Fitting atomic structures into the EM volume

High-resolution information about the domains of Endo180 is not available. Nevertheless, each domain has homology to domains of other proteins that have been solved at atomic resolution. To corroborate the quality of our EM map, we fitted the atomic structures of each domain within the EM densities (Wriggers *et al.*, 1999; Fig. 5). As interdomain linker-regions are not included in the published structures, we expected gaps that are not covered by the fitting.

The cysteine-rich domain from the mannose receptor (Liu *et al.*, 2000; Fig. 5A) fitted well into our volume (Fig. 5B). Because of the threefold symmetry of this domain, three possible solutions were found, and we selected the one in which the C terminus was located close to the FNII domain, allowing the continuity of the chain. For FNII, we used the FN 'F2 domain (Pickford *et al.*, 1997; Fig. 5B), and the fitting placed the invariant and highly conserved aromatic residues that probably interact with the non-polar residues of collagen at the side opposing the cysteine-rich domain. The atomic structure of the MBPA CTLD (Weis *et al.*, 1992) fitted well with CTLD1 (Fig. 4B). The atomic structure of the mannose receptor CTLD4 could not be fitted accurately because it contains an extended loop that projects from the CTLD core, which is proposed to be a lattice-formation artefact (Feinberg *et al.*, 2000). The best docking solution placed the N and C ends of MBPA at a position close to the N- and C-terminal connections of CTLD1, with the hydrophobic core that interacts with Ca^{2+} ions and sugars in functional C-type lectin domains facing outwards. The conformation of CTLD2 did not resemble any of the published high-resolution data for any homologous C-type lectin domain (Weis *et al.*, 1992; Feinberg *et al.*, 2000) because the N- and C-terminal ends were placed at opposite sides. The 'rigid body' docking strategy clearly cannot take into account changes in the high-resolution structure and, therefore, we could not make a proper fitting within this domain. However, a tentative docking of the MBPA atomic structure (not shown) places CTLD2 in a conformation in which the sugar-binding hydrophobic core faces the cysteine-rich domain.

Interplay among Endo180 domains

So far, three ligand-binding activities have been identified for Endo180: collagen binding to the FNII domain (Engelholm *et al.*, 2003; Wienke *et al.*, 2003), sugar binding by CTLD2 (East *et al.*, 2002), and an association with uPAR (Behrendt *et al.*, 2000). The three-dimensional volume of the N-terminal region of Endo180 shows the structural interplay between the cysteine-rich domain, the FNII domain and CTLD2, and consequently has implications for the regulation of Endo180 function. The fitting model indicates that the FNII residues involved in collagen binding will be exposed. In agreement with this, Endo180 has been shown to bind collagen/gelatin under experimental conditions compatible with the ones used in this study. Collagens can block the formation of the trimolecular uPA-uPAR-Endo180 complex (Behrendt *et al.*, 2000), and monoclonal antibodies directed against either CTLD2 and the cysteine-rich domain block Endo180-dependent cellular responses to uPA (J. Sturge, D. Wienke, L. East, G.E. Jones and C.M.I., unpublished data). The Endo180 volume provides some clues for these seemingly contradictory data. The three ligand-binding domains are connected in their three-dimensional arrangement so that events in one of the domains can modulate the structure and function of the others. We hypothesize that

Fig. 5 | Fitting the atomic structures into the Endo180 electron microscopic model. (A) Tube representation of each domain. (B) Fitting the atomic structures shown in (A) into the electron microscopic volume for sEndo180-HA-His₆ (a soluble Endo180 extracellular-domain construct that contains a carboxy-terminal haemagglutinin (HA) tag and a six-histidine (His₆) tag; shown as a black grid). CTLD, C-type lectin-like domain; Cys, cysteine-rich domain; FNII, fibronectin type II domain.

collagen binding will probably modify the conformation of the neighbouring cysteine-rich domain and the contacting CTLD2. Similarly, the conformation of CTLD2 with respect to the other domains may help to regulate the *in vivo* binding of Endo180 to physiological glycosylated ligands and, conversely, allow sugar binding to CTLD2 to modulate its interaction with the other domains. In conclusion, our data provide an important glimpse into the structural basis of how these multidomain receptors might interact and regulate the selectivity for multiple ligands.

METHODS

Sample preparation. Details of the pcDNA3-Endo180ΔCTLD3-8-Fc plasmid (East *et al.*, 2002) and the pcDNA3-ΔHindIII-sEndo180-HA-His₆ plasmid, in which the Endo180 extracellular domain was truncated at the end of CTLD8 (amino acid 1395) and fused in-frame with an HA tag, a His₆ tag and a stop codon, are available on request. Expression in COS-1 cells was performed as described previously (East *et al.*, 2002). The soluble sEndo180-HA-His₆ protein was purified on a 1-ml nickel-NTA column (Qiagen). The column was washed with equilibration buffer (50 mM NaH₂PO₄, 135 mM NaCl, 0.05% Tween 20, pH 7.3) containing 10–20 mM imidazole, and sEndo180-HA-His₆ protein was eluted sequentially with buffer containing 50 mM and 100 mM imidazole. Elution fractions were pooled, concentrated and exchanged into storage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20% (v/v) glycerol). The Endo180ΔCTLD3-8-Fc protein was purified on a HiTrap Protein G column (Amersham Biosciences) and dialysed into storage buffer. To assess the integrity of the purified protein, 5-μl aliquots were resolved by non-reducing SDS-PAGE using 10% gels,

which were either silver-stained or transferred to nitrocellulose and blotted with anti-Endo180 monoclonal antibody E1/183, anti-HA monoclonal antibody 3F10 (Roche) or an anti-human-Fc antibody (Sigma; catalogue number F2136). C-type lectin activity of purified proteins was assessed by binding to a GlcNAc-sepharose column and elution in EDTA (East et al., 2002).

Electron microscopy and three-dimensional reconstruction. A few microlitres of the sample were applied to carbon-coated grids and were negatively stained using 1% uranyl acetate. A Philips CM100 and a JEOL-1230 electron microscope, operated at 100 kV, were used to observe the samples. Micrographs were taken under low-dose conditions and were digitized using a Leafscan45 scanner (Leaf Systems, Inc.) with a 10- μ m step-size. Approximately 3,000 particles for sEndo180-HA-His₆ and 500 for Endo180 Δ CTLD3–8–Fc were initially collected. Particles were classified, and a first model was constructed and then refined as implemented in EMAN (Ludtke et al., 1999).

Fitting of the X-ray structures with the electron microscopy data. Fitting was carried out using the software SITUS (Wriggers et al., 1999). The atomic structures used were as follows: the cysteine-rich domain (mannose receptor; Protein Data Bank (PDB) reference number 1DQO), FNII domain (FN 1F2; PDB number 2FN2) and CTLD (MBPA; PDB number 2MSB).

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