A targeted deletion in the endocytic receptor gene *Endo180* results in a defect in collagen uptake

Lucy East*, Afshan McCarthy*, Dirk Wienke, Justin Sturge, Alan Ashworth & Clare M. Isacke[†]

The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK

The four members of the mannose receptor family (the mannose receptor, the M-type phospholipase A₂ receptor, DEC-205 and Endo180) share a common extracellular arrangement of an amino-terminal cysteine-rich domain followed by a fibronectin type II (FNII) domain and multiple C-type lectin-like domains (CTLDs). In addition, all have a short cytoplasmic domain, which mediates their constitutive recycling between the plasma membrane and the endosomal apparatus, suggesting that these receptors function to internalize ligands for intracellular delivery. We have generated mice with a targeted deletion of *Endo180* exons 2–6 and show that this mutation results in the efficient expression of a truncated Endo180 protein that lacks the cysteine-rich domain, the FNII domain and CTLD1. Analysis of embryonic fibroblasts reveals that this mutation does not disrupt the C-type lectin activity that is mediated by CTLD2, but results in cells that have a defect in collagen binding and internalization and an impaired migratory phenotype.

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INTRODUCTION

Proteins of the mannose receptor family have a shared structural design, but individual members have distinct ligand-binding properties and tissue-distribution patterns. Thus, although they all function, or are predicted to function, in the transport of ligands into the cell, the nature of their cargoes and the cell types in which they act are varied (East & Isacke, 2002). Endo180, the most recently identified and final member of this family, was identified independently as a novel endocytic fibroblast receptor (Isacke *et al.*, 1990; Sheikh *et al.*, 2000) and as a urokinase-type plasminogen activator receptor (uPAR)-associated protein, hence its alternative name, uPARAP (Behrendt *et al.*, 2000). In addition to its binding to uPAR, two other ligand-binding activities have been described for Endo180: Ca²⁺-dependent sugar binding and interaction with collagens.

A unique feature of the mannose receptor family is the presence of multiple C-type lectin-like domains (CTLDs) in a single polypeptide backbone. However, in the phospholipase A₂ receptor and DEC-205, none of these domains are functional, and in the

Fig. 1 | Generation of mice with a targeted deletion in *Endo180*. (A) Maps of the *Endo180* genomic locus, the targeting vector and the targeted allele. Exons are shown as black boxes; LoxP sites are shown as red arrowheads; dotted lines indicate boundaries of the 5' short-homology and 3' long-homology regions. (B) Genomic DNA isolated from mouse tail tips was digested with *Bam*HI, Southern blotted, and hybridized with a 350-bp probe located upstream of the short-homology region. B, *Bam*HI site; DT, diphtheria-toxin expression cassette; PGK-neo, phosphoglycerate-kinase-neomycin-resistance cassette.

The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK

*These authors contributed equally to this work

[†]Corresponding author: Tel: +44 20 7970 6106; Fax: +44 20 7858 3858;

E-mail: c.isacke@icr.ac.uk

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mannose receptor this activity is restricted to CTLD4 (Taylor *et al.*, 1992). Like the mannose receptor, Endo180 binds sugars with a specificity that is typical of mannose-binding C-type lectins, and again this is mediated by a single CTLD, CTLD2 (East *et al.*, 2002). The positioning of the Endo180 sugar-binding domain nearer to the amino terminus as compared with the mannose receptor has two potential consequences: first, it will be projected further from

the membrane, and may therefore be accessible to ligands that cannot interact with the mannose receptor. Second, its association with a different set of neighbouring domains may differentially modulate its activity. Evidence that Endo180 binds collagen comes from studies by Behrendt *et al.* (2000), in which it was shown that crosslinking of Endo180 and uPAR on the surface is blocked by collagens I, IV and V.

Fig. 2 | Characterization of the *Endo180* deletion. **(A)** The left panel shows the results of RT-PCR (PCR after reverse transcription) reactions from *+/+* and $\Delta Ex2-6/\Delta Ex2-6$ mouse embryonic fibroblasts (MEFs) using primers located in *Endo180* exons 1 and 10. The products were resolved by agarose-gel electrophoresis. As a control, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) PCR reactions were performed in parallel. Sequencing of the *Endo180* RT-PCR product from $\Delta Ex2-6/\Delta Ex2-6$ cells identifies messenger RNA in which exon 1 is spliced onto the start of exon 7, and exons 2–6 are absent (right panel). **(B)** Membrane preparations from MEFs were resolved by SDS-polyacrylamide gel electrophoresis using 10% gels and were western blotted either with anti-*Endo180* antibody followed by horseradish peroxidase (HRP)-conjugated anti-rabbit Ig, or with the anti-CD44 monoclonal antibody KM201 followed by HRP-conjugated anti-rat Ig (left panel). The right panel shows the structure of the wild-type *Endo180* protein and the *Endo180* protein from $\Delta Ex2-6/\Delta Ex2-6$ cells, which is missing the cysteine-rich (CR) domain, fibronectin type II (FNII) domain, C-type lectin domain 1 (CTLD1) and the first part of the CTLD1–CTLD2 linker region. The amino-terminal signal sequence is not shown. Cyto, cytoplasmic domain; TM, transmembrane domain.

Fig. 3 | Migration and chemotaxis of mouse embryonic fibroblasts expressing *Endo180* $\Delta Ex2-6$. **(A)** Wild-type (+/+) and $\Delta Ex2-6/\Delta Ex2-6$ mouse embryonic fibroblasts (MEFs) were analysed in migration assays, as described in the Methods section. The migratory paths, shown as trajectory plots, are from one, representative experiment where n was the number of cells in the experiment. The bar chart shows mean $\pm$ s.e.m. migratory speed for all cells analysed from three experiments. **(B)** Chemotactic response of +/+ and $\Delta Ex2-6/\Delta Ex2-6$ MEFs to a concentration gradient of urokinase-type plasminogen activator (uPA; 10 nM in the outer well of the Dunn chamber). The chemoattractant source is shown as a red asterisk. Migratory paths are shown as trajectory plots. The circular histograms show the proportion of cells with a migratory direction lying within each 18° interval. The red arrows represent the mean direction of migration, the green segments represent the 95% confidence interval determined by the Rayleigh test for unimodal clustering of direction. n , number of cells assayed.

In the adult, *Endo180* expression has been reported in stromal fibroblasts and in a subset of macrophages and endothelial cells (Isacke *et al.*, 1990; Schnack Nielsen *et al.*, 2002; Sheikh *et al.*, 2000). High levels of expression are also found in the embryo and neonate in chondrocytes at areas of active cartilage deposition and in tissue undergoing ossification (Engelholm *et al.*, 2001; Wu *et al.*, 1996). In addition, *Endo180* expression is upregulated in tumour endothelium and in breast cancer cells (Schnack Nielsen *et al.*, 2002; St Croix *et al.*, 2000). The expression pattern of this endocytic receptor, together with its proposed functions as a C-type lectin receptor, collagen-binding receptor and co-receptor for uPAR regulating extracellular matrix (ECM) uptake and remodelling, either by directly internalizing matrix components and/or by modulating their breakdown. To investigate further the function of this receptor, we have used a gene targeting approach to generate mice with a deletion in exons 2–6 of the *Endo180* gene.

RESULTS

Generation of mice with a targeted deletion in *Endo180*

A targeting vector was generated in which *Endo180* exons 2–5 and part of exon 6 were replaced by a phosphoglycerate-kinase-neomycin-resistance (*PGK-neo*) cassette (Fig. 1A). After transfection of R1 embryonic stem (ES) cells, a clone containing a single targeted *Endo180* allele was identified by PCR and Southern blotting. These cells were injected into blastocysts from C57BL/6J mice and transferred into pseudopregnant females. Three male chimaeras were produced, and these were crossed with C57BL/6J females to produce heterozygous animals. Heterozygotes were intercrossed to produce homozygous animals. Genotypes were identified by coat colour, PCR and Southern blotting of genomic DNA isolated from tail tips (Fig. 1B). All animals homozygous for the *Endo180* $\Delta Ex2-6$ mutation that have been born so far seem to be phenotypically normal, healthy and fertile, suggesting that this targeted deletion in *Endo180* results either in a subtle phenotype or in functional redundancy.

The targeting strategy used here leaves exon 1 (which contains the initiating methionine and N-terminal signal sequence) intact. ES cells could not be used to determine whether this results in the production of a truncated protein, as they do not express *Endo180* (data not shown). Consequently, primary mouse embryo fibroblast (MEF) cultures were established from 13.5-day (E13.5) embryos. RNA was extracted from MEFs and RT-PCR (PCR after reverse transcription) was carried out using primers to *Endo180* exons 1 and 10 (Fig. 2A). As predicted from the nucleotide sequence, a 1.7-kb fragment was amplified from wild-type MEFs. The presence of an amplified 0.7-kb product from the $\Delta Ex2-6/\Delta Ex2-6$ MEFs shows that *Endo180* messenger RNA is produced, and predicts that exon 1 is spliced onto a 3' exon that lies close to the targeted insertion. Sequencing of the 0.7-kb PCR fragment from the homozygous targeted cells identified an *Endo180* mRNA containing exon 1 spliced onto the beginning of exon 7, with a continuous open reading frame (Fig. 2A). To determine whether this transcript is efficiently translated into protein, MEFs from wild-type, heterozygous and homozygous targeted animals were analysed by western blotting using a cross-species-reactive anti-*Endo180* antibody raised against full-length, purified human *ENDO180* (Fig. 2B). In $+/+$ and $\Delta Ex2-6/\Delta Ex2-6$ MEFs, single immunoreactive bands of ~180 kDa and ~140 kDa, respectively, were detected, whereas the $+/\Delta Ex2-6$ cells expressed both forms. Hence, the targeted deletion resulted in expression of an *Endo180* protein that lacks the cysteine-rich domain (exon 2), the FNII domain (exon 3) and the first CTLD (CTLD1; exons 4–6; Fig. 2B). In addition, the size of the truncated *Endo180* product indicates that it is a transmembrane glycoprotein that has been subject to correct post-translational processing.

Characterization of *Endo180* $\Delta Ex2-6$ MEFs

Endo180 is a constitutively recycling glycoprotein (Isacke *et al.*, 1990), and we have previously reported that its internalization from the plasma membrane is dependent on a dihydrophobic motif in the cytoplasmic domain (Howard & Isacke, 2002). In permeabilized $+/+$ MEFs, *Endo180* shows a strong endosomal staining pattern (see next section), which is typical of that described for primary human fibroblasts (Isacke *et al.*, 1990). An indistinguishable staining pattern is seen in $\Delta Ex2-6/\Delta Ex2-6$ MEFs, consistent with the truncated protein having retained an intact, functional cytoplasmic domain.

It is notable that the cell-type expression of *Endo180* in both normal and diseased tissue is associated with a migratory phenotype. Consequently, $\Delta Ex2-6/\Delta Ex2-6$ MEFs were tested for their migratory ability using a Dunn chamber assay and were shown to have a significantly ($p < 0.0001$) reduced migratory speed compared with wild-type MEFs (Fig. 3A), showing that expression of *Endo180* is pro-migratory. However, full quantification of *Endo180* expression will be required to confirm that the observed reduction in migration results from loss of the *Endo180* N-terminal domains rather than from reduced receptor expression in $\Delta Ex2-6/\Delta Ex2-6$ MEFs. It has been extensively documented that interaction of uPA with its glycosylphosphatidylinositol (GPI)-anchored receptor initiates intracellular signalling events that lead to enhanced cell motility (Blasi & Carmeliet, 2002), and that presentation of a uPA concentration gradient results in cell polarization and directional cell migration (Sturge *et al.*, 2002).

Fig. 4 | The *Endo180* $\Delta Ex2-6$ mutant protein retains C-type lectin activity. Membrane preparations from $+/+$ and $\Delta Ex2-6/\Delta Ex2-6$ mouse embryonic fibroblasts were loaded onto 2-ml *N*-acetylglucosamine-sepharose columns. The columns were washed six times with 1 ml of loading buffer containing 25 mM Ca^{2+} and were eluted seven times with 1 ml of elution buffer containing 10 mM EDTA. Fractions were trichloroacetic-acid-precipitated, resolved by SDS-polyacrylamide gel electrophoresis using 10% gels and analysed by western blotting using the anti-*Endo180* antibody followed by horseradish-peroxidase-conjugated anti-rabbit Ig.

Endo180 has been identified as a uPARAP; Behrendt *et al.*, 2000), and in an independent study, we have shown that expression of *Endo180* is required for chemotaxis of cells in a uPA gradient (J.S., D.W. and C.M.I., unpublished data). Consequently, it was of interest to determine whether the $\Delta Ex2-6/\Delta Ex2-6$ MEFs showed a defect in response to uPA. When exposed to a stable gradient of uPA, wild-type MEFs showed strong chemotactic motility, but no difference in directional migration was seen with $\Delta Ex2-6/\Delta Ex2-6$ cells (Fig. 3B), suggesting that expression of the cysteine-rich domain, FNII domain and CTLD1 of *Endo180* is not required for this cellular response to uPA.

Ligand binding of *Endo180* $\Delta Ex2-6$

Endo180 is a C-type lectin that shows Ca^{2+} -dependent binding to mannose, *N*-acetylglucosamine and fucose through CTLD2 (East *et al.*, 2002). In the mannose receptor, biophysical studies and protease-resistance assays have suggested that CTLD2 is closely associated with the adjacent CTLD1 domain but is separated from CTLD3 by a flexible, exposed linker region (Napper *et al.*, 2001). Sequence comparison predicts the same arrangement for *Endo180*. Consequently, although CTLD2 is retained in the *Endo180* $\Delta Ex2-6$ protein, the absence of CTLD1 may disrupt its structure. To assess the sugar-binding properties of the *Endo180* $\Delta Ex2-6$ protein, a column-based sugar-binding assay was performed (Fig. 4). Both *Endo180* and *Endo180* $\Delta Ex2-6$ bound to the immobilized *N*-acetylglucosamine, and they showed an identical elution profile in the presence of the calcium chelator EDTA. This shows that truncation of the cysteine-rich domain, FNII domain and CTLD1 does not disrupt the folding and function of CTLD2.

To investigate whether the MEFs had a defect in collagen binding, primary cultures were incubated at 37 °C with fluorescently labelled collagen IV or denatured collagen (gelatin), and binding was monitored by fluorescence-activated cell sorting analysis. Wild-type MEFs bind both collagen IV and gelatin, but binding of collagen IV is abolished in $\Delta Ex2-6/\Delta Ex2-6$ MEFs and binding of gelatin is substantially reduced. $+/\Delta Ex2-6$ MEFs showed an

intermediate phenotype (Fig. 5A). This defect in collagen binding is not due to a downregulation of the main collagen-binding integrin subunits, β_1 and β_3 , as no difference in cell-surface integrin expression levels was detected between the $+/+$, $+/\Delta Ex2-6$ and

$\Delta Ex2-6/\Delta Ex2-6$ MEFs (Fig. 5A). Finally, Endo180 is an endocytic receptor and, consequently, it was important to assess whether wild-type MEFs were able to internalize collagens. Cells that had been incubated with labelled gelatin, as described above, were

Fig. 5 | Mouse embryonic fibroblasts expressing Endo180 $\Delta Ex2-6$ have a defect in collagen binding and uptake. Mouse embryonic fibroblasts (MEFs) were seeded into 35-mm dishes for 24 h. The cells were then incubated with OregonGreen488 (OG)–collagen-IV or OG–gelatin for 2 h. **(A)** Cells were analysed by flow cytometry. $+/+$ cells are indicated by red lines, $+/\Delta Ex2-6$ cells by green lines and $\Delta Ex2-6/\Delta Ex2-6$ cells by blue lines. In the top two graphs, black lines indicate the binding of FITC (fluorescein isothiocyanate)–BSA to $+/+$ cells. In parallel (bottom two graphs), cells were stained with an anti-integrin β_1 monoclonal antibody followed by Alexa488 anti-rat Ig, or with biotinylated anti-integrin β_3 followed by phycoerythrin–streptavidin, and were analysed by fluorescence-activated cell sorting (FACS). Black lines indicate binding of Alexa488 anti-rat Ig and PE–streptavidin alone to $+/+$ cells. **(B)** Cells were collagenase treated to remove surface-bound collagen, fixed, permeabilized and stained with anti-Endo180 followed by Alexa555 anti-rabbit Ig; nuclei were counterstained with TO-PRO-3. Scale bar, 20 μ m.

treated with collagenase to remove any surface-bound ligand, immunostained for Endo180 expression and analysed by confocal microscopy (Fig. 5B). In +/+ MEFs, internalized ligand was readily detected, whereas little or no labelled gelatin was associated with $\Delta Ex2-6/\Delta Ex2-6$ MEFs.

DISCUSSION

We provide direct evidence that Endo180 can function as a collagen receptor. Given that this activity is lost in the *Endo180* $\Delta Ex2-6$ mutant, these data support the suggestion that binding is mediated by the Endo180 FNII domain, which shares a high level of amino-acid similarity with the gelatin/collagen-binding FNII domains in fibronectin and matrix metalloproteinases 2 and 9 (Behrendt *et al.*, 2000; East & Isacke, 2002). Endo180 is an endocytic receptor and, as shown here, binding of collagens is followed by ligand internalization. A further investigation of the fate of internalized collagen showed that most of the surface-associated ligand is rapidly taken into Endo180-positive endosomes and then quickly transported into degradative intracellular compartments (Wienke *et al.*, 2003). If Endo180 is important in regulating collagen uptake and degradation, it might be expected that *Endo180* $\Delta Ex2-6$ mice would have a defect in collagen assembly/ECM organization. However, no overt phenotype has been identified in these animals. This could be due to functional redundancy in the mannose receptor family, given that other family members may also bind collagens (East & Isacke, 2002). Alternatively, there may be compensation from unrelated collagen-binding/internalization receptors; however, it should be noted that no downregulation of the main collagen-binding integrin β -subunits was detected in $\Delta Ex2-6/\Delta Ex2-6$ MEFs compared with +/+ MEFs. It will be of interest to determine whether the overexpression of Endo180 in disease pathologies results in an ECM defect in mice that contain a targeted *Endo180* deletion. In addition to a defect in collagen binding, $\Delta Ex2-6/\Delta Ex2-6$ MEFs also have an impaired migratory phenotype compared with wild-type cells. Although it is tempting to speculate that the reduced migration results from an impaired ability of the cells to interact with collagens in the Matrigel substratum, it remains a possibility that these two observations are not linked. What the data presented here do show is that collagen binding can be separated from the C-type lectin activity of Endo180.

METHODS

Construction of the targeting vector. The pKO scrambler vector (Lexicon Genetics, Inc.) was modified by inserting a diphtheria toxin (DT) expression cassette and a *PGK-neo^r* cassette. To generate an *Endo180* targeting vector, 1.5 kb of intronic sequence upstream of exon 2 was amplified by PCR from a λ PS clone and blunt-end ligated into the *HpaI* site of the modified pKO scrambler vector. A 6.5-kb 3' *Endo180* long-homology region was generated by PCR using primers within exons 6 and 10 and using 129/Sv genomic DNA as a template, and this was ligated into the *SaII* site. Full details of the targeting vector are available on request. The targeting vector was transfected into R1 ES cells, and a clone in which one copy of the *Endo180* gene had been correctly targeted was isolated and used to generate the *Endo180* $\Delta Ex2-6$ mouse, as described in the Results and Discussion section.

Generation and characterization of mouse embryonic fibroblasts.

MEFs were isolated from E13.5 embryos and cultured as described in Abbondanzo *et al.* (1993). RNA was extracted from primary MEFs using Trizol reagent (Invitrogen) and RT-PCR was performed using the Titan One Tube RT-PCR kit (Roche) with primers located in *Endo180* exon 1 (5'-GGCATCACTGTAGATCACTTGG-3') and exon 10 (5'-ATCCGAGCACAGCGCTAGGG-3'), respectively. Immunostaining was performed as described previously (Howard & Isacke, 2002) using a polyclonal anti-Endo180 antibody (Isacke *et al.*, 1990). Chemotaxis and migration were measured by direct observation and recording of cell behaviour using the Dunn migration/chemotaxis chamber as previously described (Allen *et al.*, 1998; Sturge *et al.*, 2002). MEFs were plated onto Matrigel-coated glass coverslips and cultured overnight, and, during the assay, in DME, 10% FCS. Migration was digitally recorded for 16 h at a time-lapse interval of 10 min. Chemotaxis assays were performed for 4 h in a stable concentration gradient of 10 nM uPA, with a time-lapse interval of 10 min. Further details about analysis methods are available on request. For collagen-binding assays, MEFs were seeded into 35-mm tissue culture dishes at ~50% confluency, cultured for 24 h in DME, 10% FCS, and incubated with Oregon Green (OG)-collagen IV, OG-gelatin or FITC (fluorescein isothiocyanate)-BSA (all at 20 μ g ml⁻¹) for 2 h. Cells were then washed with PBS, detached with trypsin, fixed in 1% paraformaldehyde and analysed using a Becton Dickinson FACScan flow cytometer. Alternatively, cells were treated with collagenase for 5 min at 37 °C in DME containing 0.2% type-I collagenase (Sigma; catalogue number C-0130), fixed, permeabilized, and stained with anti-Endo180 followed by an Alexa555 anti-rabbit Ig secondary antibody; nuclei were counterstained with TO-PRO-3 (Molecular Probes). Images were collected sequentially in three channels on a Leica TCS SP2 confocal microscope. For sugar-binding assays, membrane preparations were made from MEFs by homogenizing cells in 5 mM HEPES, pH 7.4, 2 mM MgCl₂. The homogenates were centrifuged at 6,400g to remove cells debris, and the supernatant was centrifuged at 14,000g to pellet the cell membranes. The resulting pellet was resuspended in 1 ml of column loading buffer and applied to a 2-ml *N*-acetylglucosamine column, as described in East *et al.* (2002).

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