

Identification and Characterization of the Endocytic Transmembrane Glycoprotein Endo180 as a Novel Collagen Receptor

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Endo180, a member of the mannose receptor family, is constitutively recycled between clathrin-coated pits on the cell surface and intracellular endosomes. Its large extracellular domain contains an N-terminal cysteine-rich domain, a single fibronectin type II domain and eight C-type lectin-like domains. The second of these lectin-like domains has been shown to mediate Ca^{2+} -dependent mannose binding. In addition, cross-linking studies have identified Endo180 as a urokinase plasminogen activator receptor-associated protein and this interaction can be blocked by collagen V. Here we demonstrate directly using *in vitro* assays, cell-based studies and tissue immunohistochemistry that Endo180 binds both to native and denatured collagens and provide evidence that this is mediated by the fibronectin type II domain. In cell culture systems, expression of Endo180 results in the rapid uptake of soluble collagens for delivery to lysosomal degradative compartments. Together with the observed restricted expression of Endo180 in both embryonic and adult tissue, we propose that Endo180 plays a physiological role in mediating collagen matrix remodelling during tissue development and homeostasis and that the observed receptor upregulation in pathological conditions may contribute to disease progression.

INTRODUCTION

Collagens represent the most abundant group of proteins in vertebrates where they play important roles in the development, morphogenesis, and growth of many tissues (Eyre, 1980; Mayne and Brewton, 1993). In addition to their mechanical properties, collagens also act as substrates for cell attachment and migration and mediate signaling events by binding to cells surface receptors such as integrins, discoidin domain receptors (DDRs), and glycoprotein VI (Vogel *et al.*, 2001).

To maintain correct tissue homeostasis, the synthesis, deposition, and degradation of collagens, which is mainly carried out by fibroblasts, must be coordinately regulated. Imbalances in collagen metabolism are manifested in pathological conditions such as inflammation, fibrosis, and tumor metastasis (Perez-Tamayo, 1978; Prockop and Kivirikko, 1995). To date, several different mechanisms have been proposed for the remodeling of existing collagen networks. Many studies have demonstrated a major function for matrix metalloproteinases (MMPs) in collagen dissolution (Lauer-Fields *et al.*, 2002). However, although MMPs have been linked to the extracellular breakdown of collagens

during inflammation (Leppert *et al.*, 2001) and cancer progression (Johansson *et al.*, 2000), an alternative intracellular degradation pathway takes place at other sites of rapid collagen turnover such as periodontium (Svoboda *et al.*, 1981) and healing wounds (McGaw and Ten Cate, 1983). This intracellular catabolytic pathway occurs predominantly via phagocytosis of collagen fibrils mediated by integrins, primarily by integrin $\alpha_2\beta_1$ with a potential regulatory contribution by integrin $\alpha_3\beta_1$. The ingested collagen is subsequently transported to lysosomes and degraded by proteases of the cathepsin family (Everts *et al.*, 1985; Arora *et al.*, 2000).

A separate tissue specific system for the uptake of denatured collagen has been described in liver endothelial cells where a collagen scavenger receptor, which only recognizes monomeric collagen α -chains but not their native triple helical form, mediates the elimination of circulating collagen waste products from the blood stream (Smedsrod *et al.*, 1985, 1990). In contrast to the integrin-mediated phagocytic route, the collagen scavenger receptor internalizes collagen via an endocytic route with subsequent delivery of the collagenous cargo from the endosomes to lysosomal compartments (Hellevik *et al.*, 1996). This demonstration of multiple pathways for collagen uptake raises two important questions: first, do different pathways exist for the internalization of different collagens, and second, although uptake pathways have been

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described in specialized tissues, are the same pathways utilized in nonspecialized tissues and in particular in stromal fibroblasts?

To address these questions, we have investigated the recently described receptor Endo180. Endo180 is the fourth and final member of the mannose receptor family, which also comprises the mannose receptor, the M-type phospholipase A₂ (PLA₂R) receptor and DEC-205 (East and Isacke, 2002). All 4 receptors contain an N-terminal cysteine-rich or ricin-type domain, a fibronectin type II (FNII) domain, 8 C-type-lectin domains (CTLDs; 10 in the case of DEC-205), a single transmembrane domain, and a short cytoplasmic domain (see Figure 1A). The proposal that Endo180 might function as a collagen receptor comes from two sources. First, FNII domains in other proteins including fibronectin and the gelatinases MMP2 and MMP9 (see Figure 1B) have been demonstrated to confer binding to denatured collagen (gelatin; Steffensen *et al.*, 1995; Pickford *et al.*, 2001). Second, Endo180 has been identified as part of a trimolecular cell surface complex together with prourokinase plasminogen activator (prouroPA) and the uPA receptor (uPAR), hence its alternative name of uPAR associated protein, uPARAP. Formation of this complex is inhibited by collagen V and to a lesser extent by collagen I and collagen IV (Behrendt *et al.*, 2000). Here we have directly determined the capacity of Endo180 to act as a collagen receptor using a combination of *in vitro* and cell-based assays.

MATERIALS AND METHODS

Reagents

Protein G-Sepharose and gelatin-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Collagen I, IV, and V, fibronectin, laminin, and vitronectin were obtained from BD Biosciences (Bedford, MA). Collagen II was from Calbiochem (San Diego, CA). TO-PRO-3, FITC-BSA, OregonGreen488-gelatin (OG-gelatin) and OregonGreen488-collagen IV (OG-collagen IV) were purchased from Molecular Probes (Eugene, OR). Collagen V was labeled using the FITC labeling kit from Calbiochem according to the manufacturer's instructions.

Antibodies and Cells

The generation of anti-human Endo180 monoclonal antibodies (mAbs) A5/158 and E1/183 and a cross-species-reactive anti-Endo180 polyclonal antiserum has been described elsewhere (Isacke *et al.*, 1990; Sheikh *et al.*, 2000). Anti-human LAMP1 mAb H4A3 was obtained from Prof. C. Hopkins (Imperial College, London). Antibodies against human integrins α_2 , α_3 , α_v , and β_1 were kindly provided by Dr. Fiona Watt (Cancer Research UK). The following collagen antibodies were purchased from DPC Biermann (Bad Nauheim, Germany): rabbit anti-collagen I (R1038), anti-collagen IV (R1041), and anti-collagen V (R1042). Horseradish peroxidase (HRP)-conjugated anti-mouse Ig was purchased from Jackson Immunoresearch (West Grove, PA). FITC anti-human Fc was purchased from Sigma (St. Louis, MO). Alexa488- and Alexa555-conjugated secondary antibodies were obtained from Molecular Probes. Cells were cultured in DME, 10% fetal calf serum, which was supplemented with 10 μ g/ml insulin for the MCF-7 cells. To generate permanently expressing lines, MCF-7 and T47D cells were transfected with the pcDNA3-Endo180 (Sheikh *et al.*, 2000), pcDNA3 Δ HindIII-Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹; Howard and Isacke, 2002), or with empty pcDNA3 vector using Lipofectamine and selection with G418 (0.5 mg/ml). Cell populations were enriched for Endo180-expressing cells by fluorescence activated cell sorting (FACS) using the anti-Endo180 mAb A5/158. For downregulation of endogenous

Endo180 the following small interfering (siRNA) oligonucleotides were used: Endo180 siRNA oligonucleotides 5'CCCAACGUCUUC-CUCAUCU dT dT^{3'} and 3'AGAUGAGGAAGACGUUGGG dT dT^{3'}; reversed Endo180 (control) siRNA oligonucleotides 5'UCUACUC-CUUCUGCAACCC dT dT^{3'} and 5'GGGUUGCAGAAGGAGUAGA dT dT^{3'}. Annealed siRNA oligonucleotides (0.5 nmol/ml) were transfected into MG-63 cells seeded onto glass coverslips or culture dishes (30–50% confluent) with 100 μ M Oligofectamine (Invitrogen, Paisley, United Kingdom) in Opti-MEM reduced serum medium (Invitrogen). Cells were incubated at 37°C for 4 h before the addition of FCS to 10% and cultured for a further 68 h.

Generation, Expression, and Purification of Endo180 Constructs

The generation of the pIgplus-Endo180 Δ CTLD5–8-Fc (East *et al.*, 2002) has been described previously. To generate the Endo180 Δ CTLD2–8-Fc construct, PCR reactions were performed with Expand long polymerase (Roche Molecular Biochemicals, Lewes, United Kingdom) and pcDNA3-Endo180 as a template with a 5' modified T7 primer (5'CTGGCTTATCGAAATTAATACGACT-CACTATAGGGA^{3'}) and the 3' primer (5'GTCTCGAGGTCTG-GAGGGGTGGGCTCGGCC^{3'}) located between CTLD1 and CTLD2, where bases underlined indicate an *Xho*I restriction site. Amplified DNA was digested with *Hind*III and *Xho*I and ligated into the pIgplus vector (R&D Systems, Abingdon, United Kingdom) digested with the same enzymes. In the pcDNA3 Δ HindIII-sEndo180-HA-HIS plasmid the Endo180 extracellular domain was truncated at the end of CTLD8 (amino acid 1395) and fused in frame with a hemagglutinin (HA) tag, a 6 \times histidine (HIS) tag, and a stop codon (Rivera-Calzada *et al.*, 2003). pIgplus-Endo180-Fc constructs and the pcDNA3 Δ HindIII-sEndo180-HA-HIS construct were expressed in COS-1 cells as previously described (East *et al.*, 2002). The supernatants were harvested after a 7-d period and loaded on a 1-ml Protein G-Sepharose column (Fc constructs) or onto a 1-ml column in which the anti-Endo180 mAb E1/183 had been coupled to cyanogen bromide-activated Sepharose (sEndo180-HA-HIS). The columns were washed with phosphate-buffered saline (PBS), and the fusion proteins were eluted with 0.1 M glycine, pH 2.5. Fractions (1 ml) were collected and neutralized with 1 M Tris-HCl, pH 9.0. Fractions containing the fusion proteins were pooled and dialyzed against PBS.

Gelatin-Sepharose Binding Assay

A gelatin-Sepharose or Sepharose column (2 ml) was equilibrated with loading buffer (50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂) and loaded with 1 ml of Endo180 Δ CTLD5–8-Fc (20 μ g/ml in loading buffer). The flow-through was collected, and the columns were washed with 5 \times 2 ml of loading buffer followed by 6 \times 1 ml of elution buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl). Fractions were precipitated by incubation with 40 μ g of bovine serum albumin (BSA) and 0.5 ml of 30% trichloroacetic acid for 30 min on ice and then centrifuged for 10 min at 4°C at 15,000 \times g. The pellets were washed twice in 1:1 ethanol ether, air-dried for 10 min, and resuspended in 40 μ l of nonreducing sample buffer. Samples (10 μ l) were resolved by 10% SDS PAGE and transferred to nitrocellulose, and Endo180 was detected using mAb A5/158 followed by HRP-conjugated anti-mouse Ig. Blots were developed using ECL reagent (Amersham Pharmacia).

Microwell Matrix Protein Binding Assay

Ninety-six-microwell plates (E.I.A./R.I.A, high binding, Costar, High Wycombe, United Kingdom) were incubated overnight at 4°C followed by 1 h at 37°C with 100 μ l of the following extracellular matrix components. Collagen type I, type II, type IV, type V, and gelatin (all at 10 μ g/ml in 10 mM acetic acid), fibronectin, laminin, vitronectin, and BSA (all at 10 μ g/ml in PBS). Plates were rinsed

three times with 0.3% BSA in PBS, blocked in the same solution for 1 h at 20°C, and then incubated with 100 μ l of Endo180 constructs (4 μ g/ml in PBS) for 1 h at 20°C. For competition experiments, stock solutions of collagen I, collagen IV, and gelatin (1 mg/ml in 10 mM acetic acid) were neutralized with 0.2 vol of 1 M Tris, pH 7.6, and preincubated in a 1:1 ratio with Endo180 constructs (8 μ g/ml in PBS). Bound Endo180 constructs were detected with 10 μ g/ml mAb E1/183 followed by 1 μ g/ml HRP anti-mouse IgG using *o*-phenylenediamine dihydrochloride (Sigma) as a substrate. The reaction was stopped by adding 3 M HCl, and the absorption was quantified in an enzyme-linked immunosorbent assay (ELISA) plate reader (Spectramax 384 plus, Molecular Devices, Wokingham, United Kingdom) at a wavelength of 492 nm. As negative controls human IgG and secondary antibody alone were used.

Flow Cytometry

Cells were harvested by exposure to trypsin/EDTA (Sigma) for 1 min at 37°C, washed in DMEM containing 10% FCS and stained on ice with 10 μ g/ml anti-Endo180 mAb A5/158 or anti-integrin mAbs for 20 min followed by 2 μ g/ml Alexa488 anti-mouse Ig for 20 min, washed twice, fixed with 1% paraformaldehyde, and analyzed on a Becton Dickinson (Oxford, United Kingdom) FACScan flow cytometer. For the collagen-binding studies, cells were seeded on 6-well tissue culture dishes and grown to 70–90% confluency. Cells were starved for 1 h with serum-free DME and then incubated with OG-gelatin, OG-collagen IV, FITC-collagen V, or FITC-BSA at 20 μ g/ml in serum-free DME for 2 h. Cells were then washed with PBS, harvested, fixed, and FACS-analyzed as above.

Immunostaining

Cells seeded on glass coverslips were stained for cell surface Endo180 by incubation with 10 μ g/ml mAb A5/158 at 4°C for 60 min. Cells were then fixed for 20 min with 3% paraformaldehyde, permeabilized with 0.2% saponin, and incubated for 45 min with 2 μ g/ml Alexa555 anti-mouse Ig. For intracellular staining, cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% saponin before incubation with primary and secondary antibodies. Nuclei were counterstained with TO-PRO-3 (Molecular Probes) and images were collected sequentially in three channels on a Leica TCS SP2 confocal microscope (Milton Keynes, United Kingdom). For immunohistochemistry of murine kidney, 25- μ m Vibratome sections were cut from 4% paraformaldehyde fixed (1 h) adult female C3H murine kidneys. Sections were then permeabilized with 0.5% Triton X-100 for 30 min, blocked for 30 min with immunofluorescence buffer (PBS containing 1% BSA and 2% FCS), and incubated for 3 h at 37°C in 100 μ l of Endo180 Δ CTLD5–8-Fc (100 μ g/ml in immunofluorescence buffer) followed by FITC anti-human Fc for 1 h at room temperature. Nuclei were counterstained with TO-PRO-3 and sections were dried onto poly-D-lysine-coated glass slides. Where indicated, sections were double-labeled with anti-collagen antibodies followed by Alexa555 anti-rabbit Ig. For controls, sections were pretreated for 5 min at 37°C with DME containing 0.2% type I collagenase (Sigma C-0130) or probed with Endo180 Δ CTLD5–8-Fc that had been preincubated with 2 μ g/ml collagen I (37°C, 1 h). For immunohistochemistry of human breast, 7- μ m cryosections were cut from normal adult human breast and stored at –80°C. Sections were thawed, fixed in 4% paraformaldehyde for 40 min, and then stained with anti-Endo180 mAb A5/158 and anti-collagen I antibody followed by Alexa555 anti-mouse Ig and Alexa488 anti-rabbit Ig and counterstained with TO-PRO-3.

RESULTS

Endo180 Binds to Collagens

The FNII domain is the most highly conserved (44–63% sequence identity) domain within members of the mannose

receptor family and shares 45 and 42% sequence identity with the gelatin/collagen-binding FN-1F2 and FN-2F2 domains of fibronectin, respectively (Figure 1B). Sequence analysis of this domain in Endo180 shows that it has conserved all but one of the amino acid residues found to be essential for the gelatin-binding activity of MMP9 (Collier *et al.*, 1992), making gelatin/collagen an obvious candidate for an Endo180 ligand. To investigate a possible interaction between Endo180 and collagen, we generated a series of soluble Endo180 extracellular domain constructs fused either to an HA-HIS tag or to the Fc portion of human IgG1 (Figure 1A) and expressed these fusion proteins in COS-1 cells (Figure 2A). In initial studies, a fusion protein deleted after CTLD4 (Endo180 Δ CTLD5–8-Fc) was tested for its ability to bind to gelatin (denatured collagen I) immobilized on Sepharose. The results show for the first time a direct interaction between Endo180 and denatured collagens.

To determine the binding specificity of this construct, ELISA assays were performed with gelatin, collagens, and other extracellular matrix components as substrates. Endo180 Δ CTLD5–8-Fc bound to gelatin and the four different collagen types tested (type I, type II, type IV, and type V), whereas only background binding was detected on fibronectin, laminin, or vitronectin. The binding to gelatin could be substantially competed by collagen I or collagen IV. Conversely, collagen IV binding was reduced to background levels by preincubation of the Endo180 Δ CTLD5–8-Fc with gelatin, demonstrating that binding of collagens and gelatin can be cross competed and does not require CTLDs 5–8. However, this fusion protein retains CTLD2 which has previously been demonstrated to mediate the Ca²⁺-dependent Endo180 sugar binding (East *et al.*, 2002).

To further define the collagen-binding domain in Endo180, parallel assays were performed with Endo180 Δ CTLD2–8-Fc in which CTLD2 had been deleted (Figure 1A). Endo180 Δ CTLD2–8-Fc showed a similar binding profile to Endo180 Δ CTLD5–8-Fc, with the exception that binding to collagen IV was reduced (Figure 3B). In addition, Endo180 Δ CTLD5–8-Fc collagen binding was retained in the presence of the calcium chelator EDTA (our unpublished results), indicating that collagen binding is distinct from Endo180 C-type lectin activity. Finally, these binding data are not an artifact arising from the use of truncated Endo180 constructs or due to the presence of an Fc tail, which results in dimerization of the fusion proteins (Figure 2A) as an independent monomeric construct containing the entire Endo180 extracellular domain, sEndo180-HA-HIS (Figure 1A), bound to gelatin and collagens but not other extracellular matrix components, and again the gelatin and collagen binding could be cross-competed (Figure 3C). These data provide further evidence that in common with equivalent domains from fibronectin, MMP2, and MMP9, it is the Endo180 FNII domain that mediates the observed collagen binding. However, because an Fc-construct containing only the cysteine-rich and FNII domains is unstable, the possibility that the FNII neighboring domains contribute to collagen binding cannot be excluded.

Endo180 Functions in Cells as a Collagen Binding and Internalization Receptor

To study the binding of collagen to cellular Endo180, we screened several lines for their Endo180 expression and

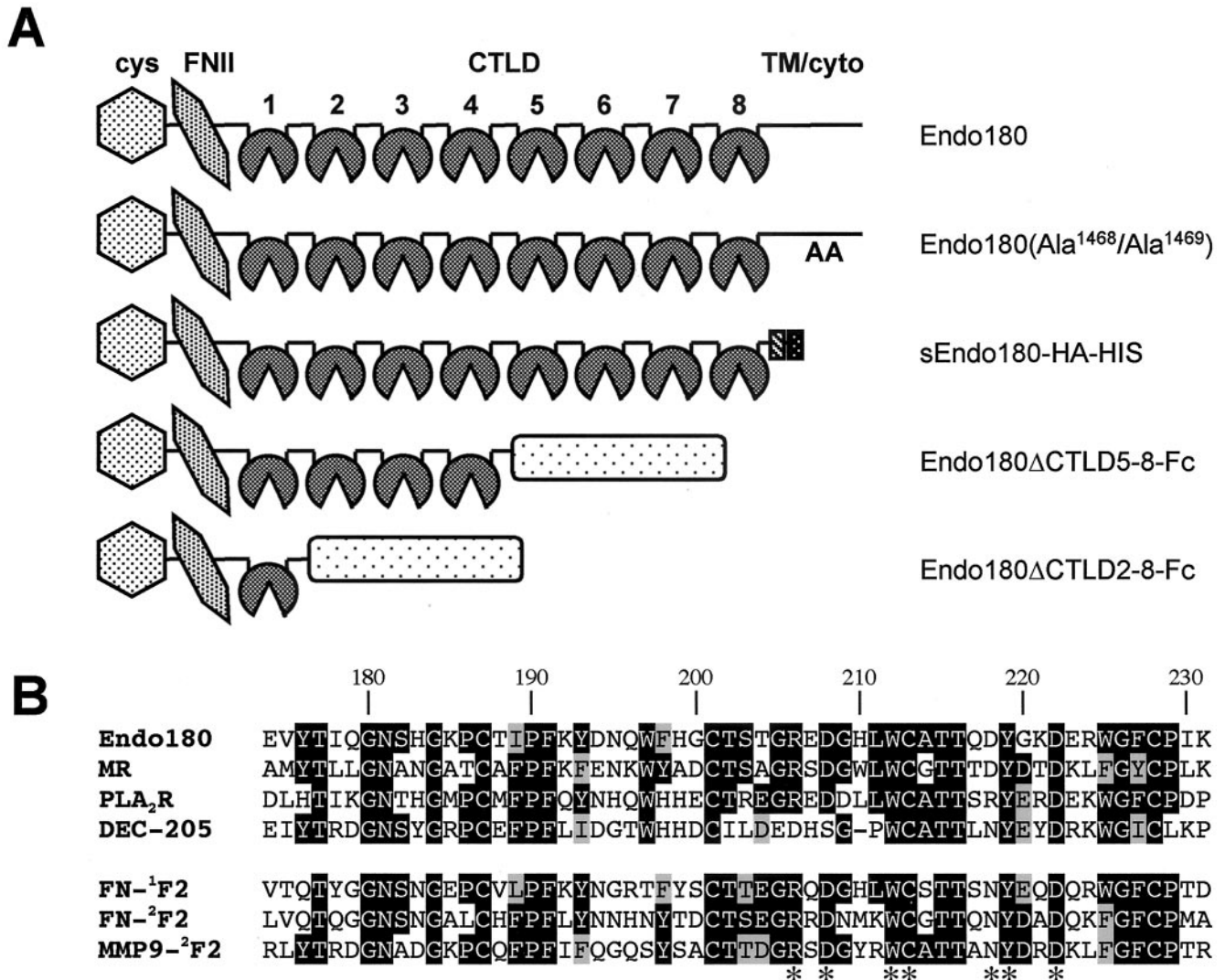


Figure 1. Schematic representation of Endo180 constructs and sequence comparison of fibronectin-type II (FNII) domains. (A) Structure of Endo180 showing the FNII domain located between the cysteine-rich domain (cys) and eight C-type lectin-like domains (CTLD). In the Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) construct the leucine-valine endocytosis motif in the cytoplasmic domain (cyto) has been mutated to two alanine residues (Howard and Isacke, 2002). Soluble constructs were generated in which the Endo180 extracellular domain was fused in frame with a hemagglutinin and histidine tag (sEndo180-HA-HIS) or in which Endo180 truncated after CTLD4 (Endo180ΔCTL5-8-Fc) or after CTLD1 (Endo180ΔCTL2-8-Fc) was fused in frame with the Fc tail of human IgG1. (B) The FNII domain of human Endo180 is aligned with the FNII domains of the other members of the mannose receptor family (MR, mannose receptor; PLA₂R, phospholipase A₂ receptor), FN⁻¹F2 and FN⁻²F2, first and second FNII domains of fibronectin; MMP9⁻²F2, second FNII domain of matrix metalloproteinase 9. Numbering refers to the amino acid sequence of human Endo180. Identical residues and residues with conservative changes are shown in black and gray, respectively. Residues found to be important for gelatin binding in MMP9 are marked with an asterisk.

found the breast cancer cell line MCF-7 to be Endo180 negative. MCF-7 cells were transfected with a wild-type Endo180 construct, with an Endo180 mutant in which the dihydrophobic Leu¹⁴⁶⁸/Val¹⁴⁶⁹ endocytosis motif in the cytoplasmic domain had been mutated to alanines, Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) (Howard and Isacke, 2002), and with vector alone. Characterization of these transfected populations by Western blotting confirmed that wild-type Endo180 and Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) were expressed at the correct molecular weight of 180 kDa (Figure 4A).

Immunofluorescence microscopy revealed that their distribution was as previously reported in transfected fibroblasts (Howard and Isacke, 2002; see Figures 5 and 8A) in that wild-type Endo180 was localized in a punctate distribution on the cell surface and in intracellular endosomes, whereas the nonendocytic Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) mutant was exclusively found on the cell surface. FACS was used to obtain cell populations in which at least 80% of the cells stably expressed the appropriate construct (Figure 4B).

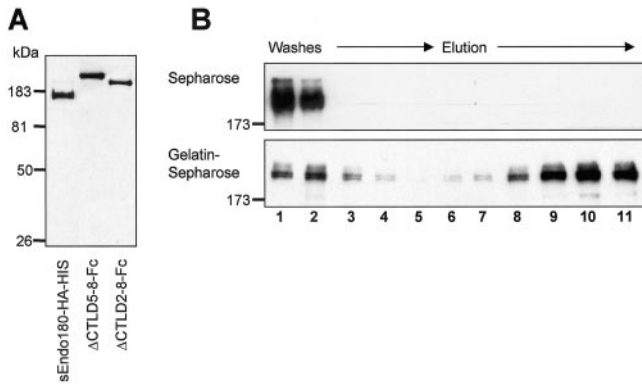


Figure 2. Soluble Endo180 binds denatured collagen. (A) Purified sEndo180-HA-HIS, Endo180 Δ CTLD5-8-Fc, and Endo180 Δ CTLD2-8-Fc were resolved by 8% nonreducing SDS PAGE and blotted onto nitrocellulose. The blot was probed with the anti-Endo180 mAb E1/183 followed by HRP anti-mouse Ig and ECL detection. Note the size of the Fc constructs reflects their Fc-mediated dimerization. (B) Endo180 Δ CTLD5-8-Fc (20 μ g) was loaded onto Sepharose or gelatin-Sepharose columns, and wash (1–5) and elution (6–11) fractions were analyzed by Western blotting as described in MATERIALS AND METHODS.

To determine their collagen-binding ability, transfected MCF-7 cells were incubated for 2 h at 37°C with OregonGreen488(OG)-gelatin, OG-collagen IV, FITC-collagen V, or FITC-BSA. Analysis of the cells by flow cytometry revealed that significantly more gelatin was associated with the wild-type Endo180-expressing MCF-7 cells than with vector alone-transfected cells (Figure 4C). This was not due to a nonspecific increase in pinocytotic or endocytotic activity because both Endo180 and vector alone-transfected cells

show an equivalent background level of FITC-BSA binding (Figure 4, C and D, and our unpublished results). A similar binding profile was observed with OG-collagen IV (Figure 4D) and FITC-collagen V (our unpublished results). Moreover, unlabeled gelatin was shown to effectively block OG-collagen IV binding, whereas both collagen I and collagen IV could compete for OG-gelatin binding (our unpublished results). Interestingly, MCF-7 cells expressing Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) showed higher levels of cell surface receptor but lower levels of associated gelatin and collagen than wild-type Endo180-expressing cells. To determine whether this difference in binding reflected the ability of wild-type Endo180, but not Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹), to internalize and thereby accumulate collagens, cells incubated with OG-gelatin were stained for Endo180 and examined by confocal microscopy (Figure 5). No Endo180 expression or OG-gelatin binding was detected in vector alone-transfected cells. In sharp contrast, wild-type Endo180-expressing cells showed strong gelatin staining distributed in a vesicular pattern throughout the cells. Cross-sectional analysis of confocal stacks clearly revealed the gelatin localized intracellularly. MCF-7 cells expressing the nonendocytic Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) mutant also showed strong binding of gelatin. However, in both horizontal and vertical sections, this gelatin was exclusively colocalized with Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) at the cell surface.

To confirm that these results were due to a specific interaction between collagens and cellular Endo180, the following control experiments were undertaken. First, the possibility that collagen binding in Endo180-expressing cells resulted from an upregulation of other collagen receptors was addressed. In the majority of cell types, the principal collagen receptor is integrin $\alpha_2\beta_1$ (Lee *et al.*, 1996) although integrins α_1 , α_3 , and α_V also have collagen-binding activity (Davis, 1992). By flow cytometry analysis, cell surface expression of integrins α_2 , α_3 , α_V , and β_1 was not changed

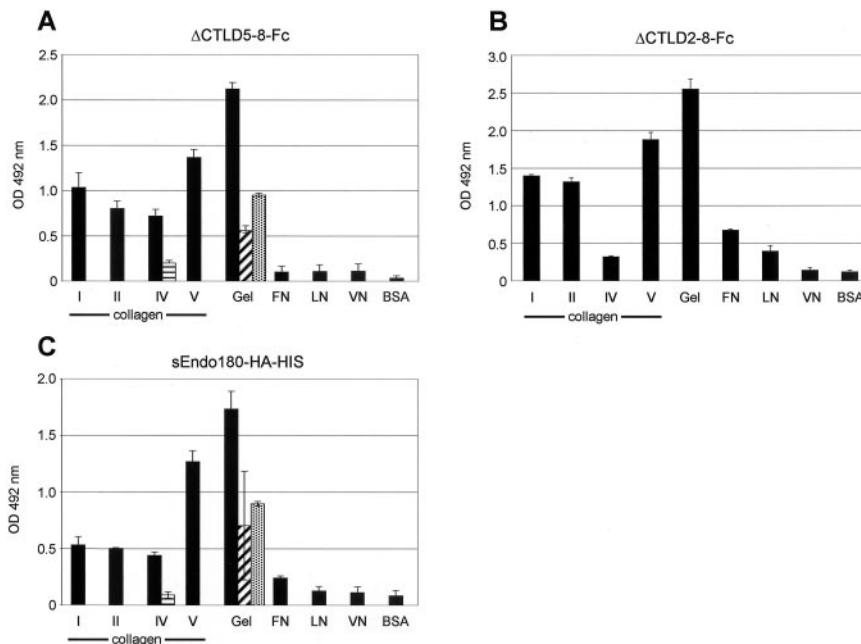


Figure 3. Binding specificity of soluble Endo180. Collagens I, II, IV, V, gelatin, fibronectin (FN), laminin (LN), vitronectin (VN), or BSA were immobilized onto multititer plates, incubated with Endo180 Δ CTLD5-8-Fc (A), Endo180 Δ CTLD2-8 (B), or sEndo180-HA-HIS followed by Endo180 mAb E1/183 and HRP anti-mouse Ig. The amount of bound construct was quantified using the OPD substrate on an absorbance plate reader set at 492 nm. The effects of preincubation of the constructs with collagens is as follows: gelatin competition for collagen IV binding (horizontal striped bars), collagen I competition for gelatin binding (hatched bars), collagen IV competition for gelatin binding (stippled bars). Values are the mean results from triplicate samples \pm SD. Equivalent results were obtained in at least three separate experiments.

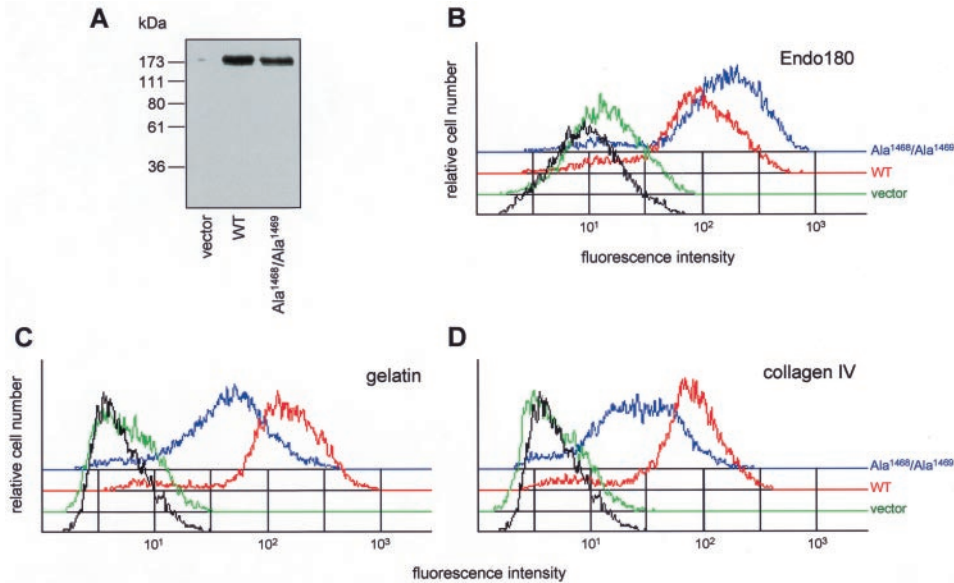
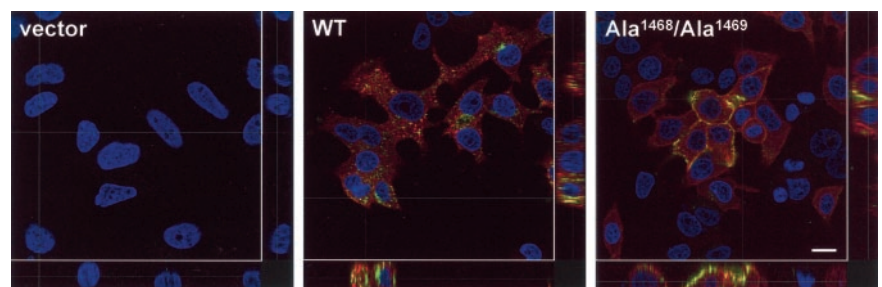


Figure 4. Collagen binding by MCF-7 cells transfected with Endo180. (A) MCF-7 cells transfected with wild-type (WT) Endo180, Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹), or with pCDNA3 vector alone were subject to Western blot analysis using anti-Endo180 mAb E1/183 followed by HRP anti-mouse Ig. (B) In parallel, cells were stained with mAb E1/183 followed by Alexa488 anti-mouse Ig and analyzed by FACS. Black lines show binding of second antibody alone to WT Endo180-transfected cells. Eighty-two percent and 88% of the cells expressed WT Endo180 and Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹), respectively. To assess collagen binding, MCF-7 cells expressing wild-type Endo180 (red), Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) (blue), or vector alone (green) seeded for 2 d were incubated with OregonGreen488 (OG)-gelatin (C) or OG-collagen IV (D) in serum-free DME for 2 h at 37°C. Cells were then washed, detached, and subject to FACS analysis. Black lines indicate binding of FITC-BSA to wild-type Endo180-expressing cells.

after transfection with either wild-type Endo180 or Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹; Figure 6). Although it cannot be ruled out that Endo180 expression results in upregulation of a nonintegrin collagen receptor such as DDR1 (Vogel *et al.*, 2000), these data demonstrate that Endo180 does not have a nonspecific effect on cell surface expression of other transmembrane receptors. Second, collagen-binding experiments were repeated in an independent cell line. The T47D mammary carcinoma cells have low levels of endogenous Endo180 expression and bind correspondingly low levels of gelatin and collagen IV (Figure 7A). T47D cells were transfected with Endo180 and expression confirmed by flow cytometry (Figure 7A), Western blotting, and immunofluorescence microscopy (our unpublished results). In a manner similar to that described for MCF-7 cells, transfection of exogenous Endo180 in T47D cells strongly enhanced the association with gelatin and

collagen IV. Third, it was important to establish that endogenous Endo180 could mediate collagen binding and that the results obtained with the MCF-7 cells and T47D cells were not an artifact of the transfection system. As previously described (Howard and Isacke, 2002), the osteosarcoma cell line MG-63 is Endo180 positive (Figure 7B), and although levels of expression are lower than observed in the transfected populations, these cells showed strong gelatin and collagen binding. Using siRNA, the expression of Endo180 in MG-63 cells could be markedly reduced compared with cells treated with control siRNA oligonucleotides. This effect was specific for Endo180 in that the control siRNA oligonucleotides had no effect on Endo180 expression, whereas the Endo180 siRNA oligonucleotides had no effect on expression of integrins α_2 , α_3 , α_V , and β_1 . In agreement with the data obtained with transfected MCF-7 cells (Figure 4), downregulation of Endo180 almost completely

Figure 5. Endo180 mediates uptake of collagen. MCF-7 cells transfected with vector alone, WT Endo180, or Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) and incubated with OG-gelatin (green) as described in Figure 4 were fixed, permeabilized, and stained with anti-Endo180 mAb A5/158 followed by Alexa555 anti-mouse Ig (red), and nuclei were counterstained with TO-PRO-3 (blue). Confocal *xy* horizontal sections are shown in main panels, and *xz* and *yz* vertical sections are shown to the right and below, respectively. Bar, 20 μ m.



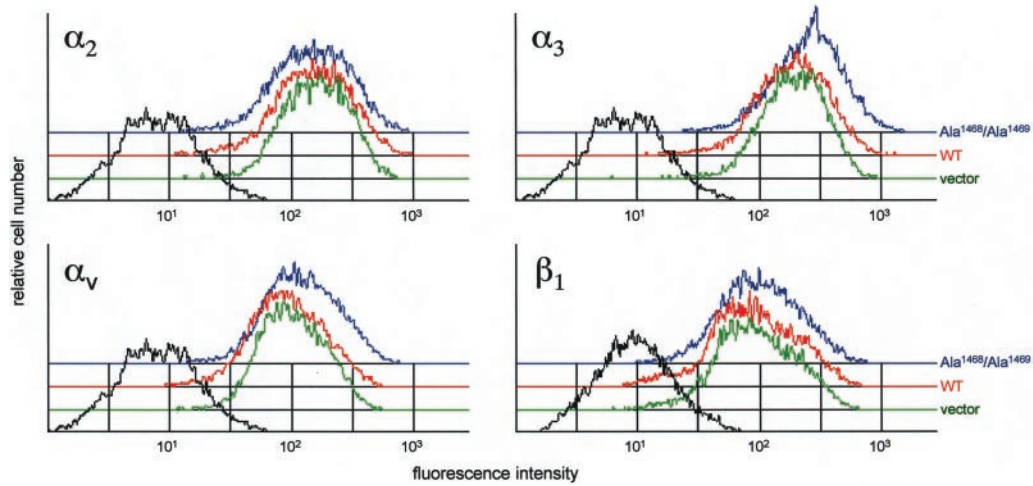


Figure 6. Expression of Endo180 in MCF-7 cells does not alter cell surface integrin levels. MCF-7 cells expressing wild-type Endo180 (red), Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) (blue), or vector alone (green) were incubated with mAbs directed against α_2 , α_3 , α_v integrins followed by Alexa488 anti-mouse Ig or with a FITC-conjugated anti- β_1 integrin mAb and were FACS-analyzed. Profiles in black represent wild-type Endo180-expressing cells incubated with second antibody alone (α_2 , α_3 , α_v) or no antibody (β_1).

abolished the ability of the MG-63 cells to bind gelatin and collagen (Figure 7B). Identical results were obtained by siRNA treatment of the Endo180-expressing HT1080 fibrosarcoma cells (our unpublished results). Together these data confirm that Endo180, both exogenous and endogenous, can act as a collagen-binding receptor and that the endocytic activity of Endo180 is required for collagen internalization.

Collagen is Rapidly Internalized by Endo180 into Early Endosomes and Subsequently Transported to Lysosomes

Endo180 is rapidly and constitutively internalized from the cell surface, cycled through early endosomes, and returned to the plasma membrane (Isacke *et al.*, 1990; Howard and

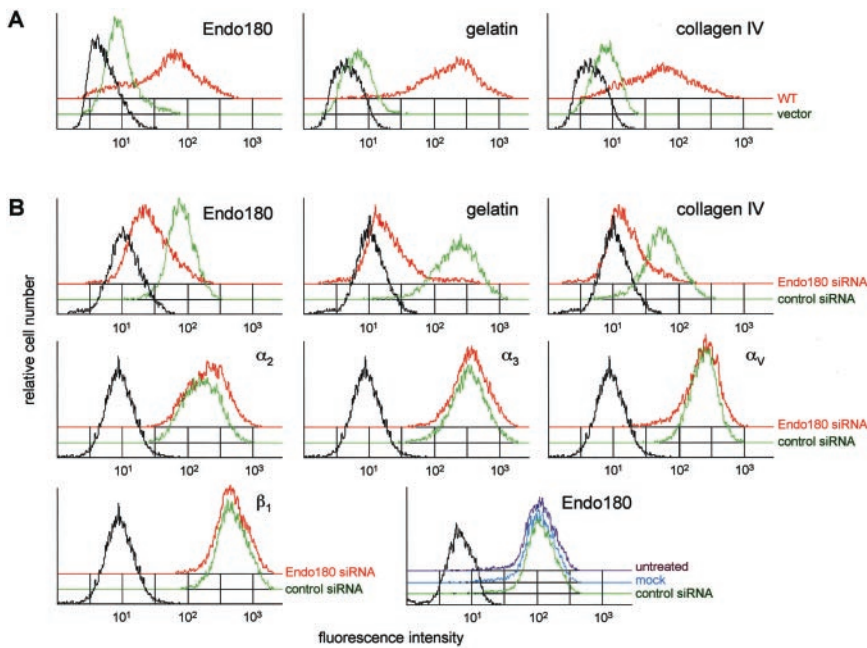


Figure 7. Expression of Endo180 in T47D breast carcinoma cells results in collagen binding, whereas downregulation of endogenous Endo180 in MG-63 cells reduces collagen binding. (A) T47D cells were transfected with vector alone (green) or wild-type Endo180 (red), subjected to one round of cell sorting, and analyzed by FACS using anti-Endo180 mAb E1/183 followed by Alexa488 anti-mouse Ig. Seventy-seven percent of the latter cells expressed Endo180. Black line indicates binding of second antibody alone to Endo180-expressing cells. To assess collagen binding, T47D cells seeded for 2 d were incubated with OG-gelatin or OG-collagen IV in serum-free DME for 2 h at 37°C. Cells were then washed, detached, and subjected to FACS analysis. Black lines indicate binding of FITC-BSA to Endo180-expressing cells. (B) MG-63 cells treated for 72 h with Endo180 siRNA oligonucleotides or control siRNA oligonucleotides were assayed for Endo180 expression and gelatin or collagen IV binding as described for T47D cells. FACS analysis of integrin expression was as in Figure 6. To confirm that control siRNA treatment did not alter Endo180 expression, parallel dishes of cells were mock-transfected with Oligofectamine or left untreated. Black lines indicate binding of second antibody alone or FITC-BSA to control cells.

Isacke, 2002). To examine the intracellular trafficking of collagens, pulse chase experiments in conjunction with confocal microscopy were undertaken using the transfected MCF-7 cells. For these experiments, OG-gelatin was bound to the cells at 4°C to prevent ligand internalization. Excess unbound gelatin was removed by washing, and the cells were either fixed (time 0) or warmed to 37°C for up to 4 h. At the end of the experiment cells were stained with antibodies against Endo180 or the lysosomal protein LAMP1. After binding at 4°C, OG-gelatin was restricted to the cell surface in both wild-type Endo180-expressing cells (Figure 8A) and Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹)-expressing cells (our unpublished results). In cells in which an anti-Endo180 mAb was incubated together with the OG-gelatin to stain cell surface Endo180, colocalization of ligand and receptor in a punctate pattern typical of clathrin-coated pits was observed (Figure 8A, top panel).

In parallel samples in which the cells were permeabilized before staining, a characteristic strong early endosomal staining of Endo180 was observed but no internalized gelatin was detected (Figure 8A, bottom panel). After a chase period of 5 min at 37°C the cell surface localized gelatin had largely disappeared and instead was found in fairly homogeneous vesicular structures throughout the cytoplasm. Almost all of the internalized gelatin appeared in vesicles that were costained for Endo180 (Figure 8B top panel), and no gelatin was detected in lysosomal compartments as identified with the anti-LAMP1 mAb (Figure 8B, bottom panel). An identical pattern was observed with OG-collagen IV (Figure 8B, top panel and inset), demonstrating that Endo180 is able to mediate early endosomal delivery of native as well as denatured collagen. After 20 min chase the gelatin-containing vesicles became more concentrated to a perinuclear localization and showed no colocalization with either the Endo180-positive early endosomes or LAMP1-positive lysosomes (our unpublished results). This indicates that the association between Endo180 and gelatin is transient and that the collagenous ligand is rapidly transferred to Endo180-negative late endosomal compartments. Because no reduction in Endo180 staining was observed in these cells, it is likely that this receptor is recycled to the plasma membrane.

Over the next few hours, the distribution of internalized gelatin changed dramatically. First, the amount of internalized gelatin was variable with many cells having only very low levels of fluorescence. Second, in cells where gelatin could be readily detectable, it was predominantly localized in less abundant but larger and more heterogeneous vesicles, many of which had a typical ring-like LAMP1 staining (Figure 8C, bottom panel and inset). However, it should be noted that the overlap between gelatin and LAMP1 at 4 h was not complete, and a significant number of gelatin-positive vesicles were neither stained with LAMP1 nor with Endo180 antibodies (Figure 8C, top and bottom panels). In the Endo180(Ala¹⁴⁶⁸/Ala¹⁴⁶⁹) transfected cells, even after 4 h at 37°C, OG-gelatin and OG-collagen IV were still detected at the cell surface, and no internalized ligand was observed (our unpublished results).

Endo180 Binds Native Collagens

The results presented so far have demonstrated that Endo180, either endogenous or transfected, can act as a

receptor for commercially obtained solubilized collagens. To confirm that Endo180 can interact with native collagens, tissue sections were probed with the Endo180 Δ CTLD5–8-Fc chimera. Sections from murine kidney were used in this analysis because it had previously been demonstrated that Endo180 expression in this tissue was restricted to the interstitial fibroblasts and that the epithelial cells were receptor negative (Isacke *et al.*, 1990). Similarly, the distribution of collagens in the kidney has been well documented (Roll *et al.*, 1980; Martinez-Hernandez *et al.*, 1982; Yoshioka *et al.*, 1990). Probing these sections with the Endo180 Δ CTLD5–8-Fc chimera revealed that ligand binding was localized to the renal interstitium in the cortex, with no staining within the tubules (Figure 9A). As a control, no staining was observed in parallel sections where the Endo180-Fc chimera was replaced with human IgG (our unpublished results). Binding of the Endo180-Fc fusion protein was blocked by preincubation with collagen I (Figure 9B) or by pretreatment of the sections with collagenase (Figure 9C), indicating that the predominant tissue staining observed with soluble Endo180 constructs results from binding to native collagens. This was further confirmed by the strong codistribution of Endo180 Δ CTLD5–8-Fc with fibrillar collagen I in the interstitial stroma (Figure 9, D–F). Similarly, there was a considerable degree of overlap in staining between the Endo180-Fc chimera with both the basement membrane collagen IV and the interstitial collagen V (Figure 9, G and H). Finally, to examine the distribution of Endo180 and collagens *in vivo*, sections of human breast were doubled labeled with anti-Endo180 mAb A5/158 and anti-collagen I antibody (Figure 9, I–K). Similar to the distribution in the kidney (Isacke *et al.*, 1990), Endo180 is not expressed on the luminal or myoepithelial cells lining the breast ducts and lobules but is strongly expressed by stromal fibroblasts. The Endo180-positive intralobular fibroblasts project extensions to essentially encompass the ducts and lobules (Figure 9I) and contact both the collagen IV containing basement membrane (our unpublished results) and interstitial collagen I-containing matrix (Figure 9J). Similarly, elongated Endo180-positive interlobular fibroblasts are observed aligned with the collagen I containing fibers.

DISCUSSION

In *in vitro* assays Endo180 binds gelatin and collagens I, II, IV, and V, and because the binding of gelatin and collagen both to soluble and cellular Endo180 can be cross-competed, it suggests that there is a common binding site for these ligands. Within the limits of these assays, it was observed that Endo180 bound more efficiently to gelatin than to collagen. The reason for this is not known but it may reflect a higher affinity of Endo180 for denatured collagens or simply the availability of more Endo180 binding sites in gelatin. Within Endo180, the gelatin/collagen-binding site has been localized to a fragment containing the N-terminal cysteine-rich, FNII and CTLD1 domains and given that this FNII domain has sequence conservation with other collagen-binding FNII domains, we predict that it is this domain that is primarily responsible for the interaction with ligand. In support of this, cells expressing an Endo180 mutant in which the three N-terminal domains have been deleted show a collagen-binding and internalization defect (East *et al.*, 2003).

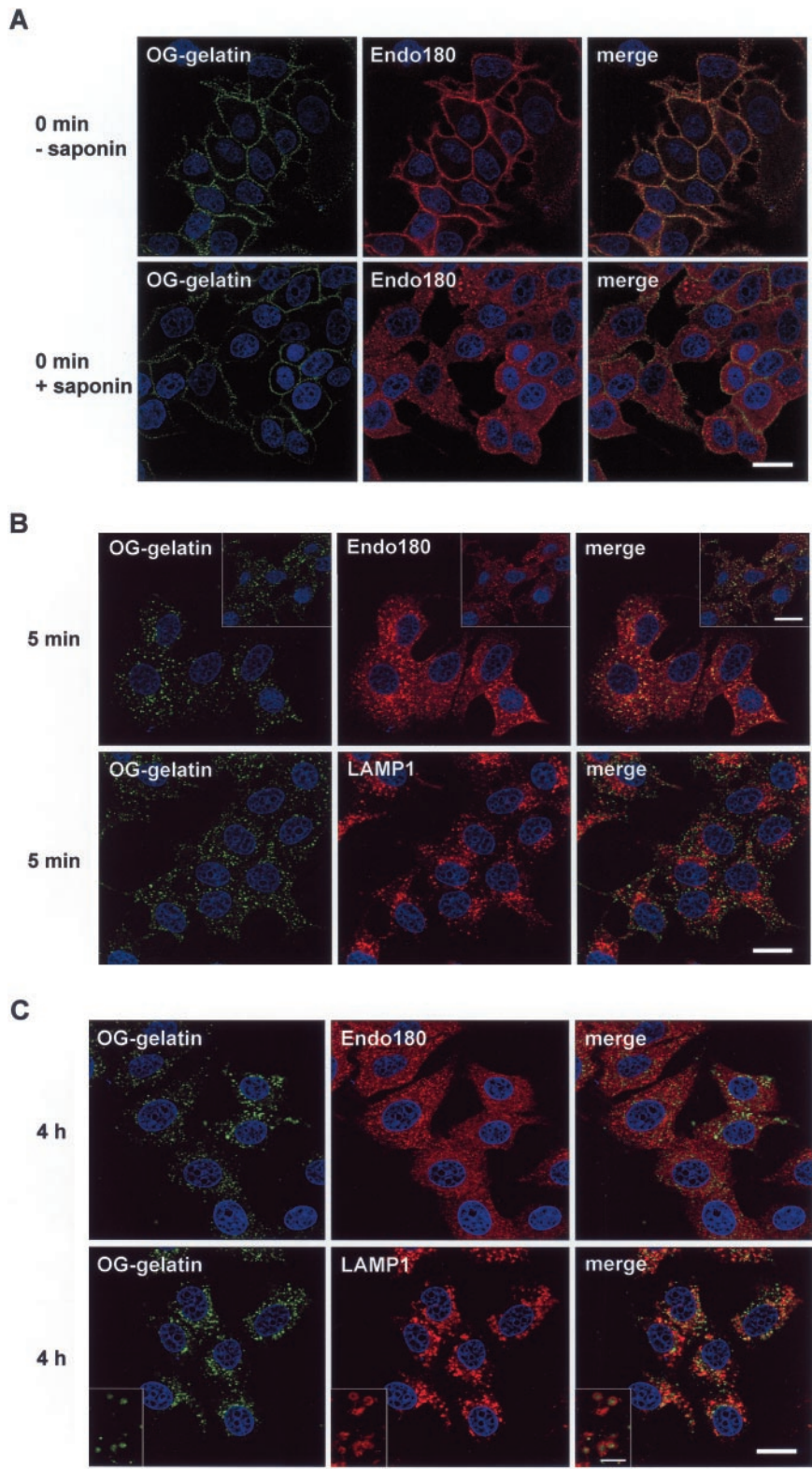


Figure 8. Distribution of gelatin in Endo180-expressing MCF-7 cells. MCF-7 cells expressing wild-type Endo180 were incubated with OG-gelatin or OG-collagen IV (green) at 4°C and then warmed to 37°C as described in MATERIALS AND METHODS. At times indicated cells were stained with anti-Endo180 mAb A5/158 or anti-LAMP1 mAb H4A3 followed by Alexa555 anti-mouse Ig (red), and the nuclei were counterstained with TO-PRO-3 (blue) and analyzed by confocal microscopy. (A) Cells were either incubated with anti-Endo180 mAb together with OG-gelatin at 4°C for 1 h and then fixed, permeabilized, and incubated with Alexa555 anti-mouse Ig (top panels) or incubated with OG-gelatin at 4°C for 1 h, fixed, permeabilized and then stained for Endo180 (bottom panels). (B) Cells warmed to 37°C for 5 min were fixed, permeabilized, and stained for Endo180 (top panels) or LAMP1 (bottom panels). Insets in top panels show parallel cultures incubated with OG-collagen IV and stained for Endo180. (C) Cells warmed to 37°C for 4 h were fixed, permeabilized, and stained for Endo180 (top panels) or LAMP1 (bottom panels). Insets in bottom panels show higher magnification images of main panel. Bar, 20 μm except inset in C where bar, 4 μm .

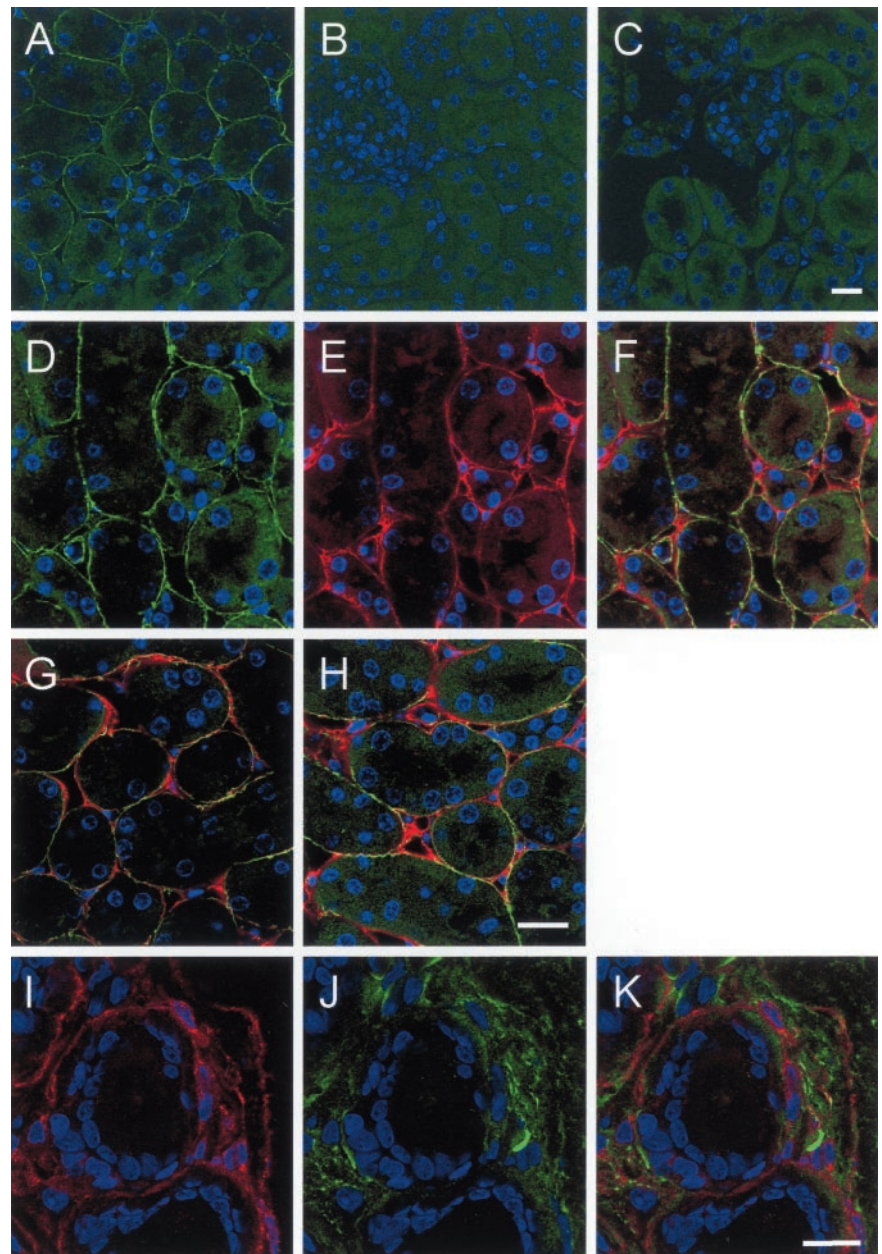


Figure 9. Distribution of Endo180 and Endo180 ligand in vivo. (A–H) Paraformaldehyde fixed murine kidney sections, 25 μm , were probed with (A) Endo180 $\Delta\text{CTL D5-8-Fc}$ followed by FITC anti-human Fc or (B) Endo180 $\Delta\text{CTL D5-8-Fc}$ preincubated with collagen I. (C) Binding of Endo180 $\Delta\text{CTL D5-8-Fc}$ to sections that had been pretreated with collagenase. (D–F) Sections that were incubated with Endo180 $\Delta\text{CTL D5-8-Fc}$ followed by FITC anti-human Fc (green) and then with anti-collagen antibody followed by Alexa555 anti-rabbit Ig (red). Nuclei were counterstained with TO-PRO-3 (blue). (D) Endo180 $\Delta\text{CTL D5-8-Fc}$; (E) anti-collagen I; (F) merged image. (G) Merged image of Endo180 $\Delta\text{CTL D5-8-Fc}$ and anti-collagen IV; (H) merged image of Endo180 $\Delta\text{CTL D5-8-Fc}$ and anticollagen V. (I–K) Human breast cyrosections, 7 μm , were fixed and probed with (I) anti-Endo180 mAb A5/158 and Alexa555 anti-mouse Ig, followed by (J) anti-collagen I and Alexa488 anti-rabbit Ig. (K) Merged imaged. Bar, 20 μm .

The principal difference from other gelatin/collagen-binding proteins is that Endo180 only contains a single FNII domain. In fibronectin, the 42-kDa gelatin binding fragment ($^6\text{F1}^1\text{F2}^2\text{F2}^7\text{F1}^8\text{F1}^9\text{F1}$) contains two FNII domains, $^1\text{F2}$ and $^2\text{F2}$. Mapping of the gelatin-binding site within this fragment produced conflicting results (Owens and Baralle, 1986; Ingham *et al.*, 1989; Skorstengaard *et al.*, 1994; Pickford *et al.*, 2001), most probably because the $^6\text{F1}^1\text{F2}^2\text{F2}$ region forms a hairpin loop with extensive interaction between the $^6\text{F1}$ and $^2\text{F2}$ domains (Pickford *et al.*, 2001) and as a consequence, loss of neighboring domains or expression of individual domains would disrupt the FNII domain structure and hence the collagen-binding properties. Moreover, in a recent study,

each of the six domains within the gelatin-binding fragment were deleted either alone or in combination. Interestingly all six domains were shown to contribute to ligand-binding affinity, suggesting that the role of domain:domain interactions extends beyond the $^6\text{F1}^1\text{F2}^2\text{F2}$ region (Katagiri *et al.*, 2003). In MMP2 each of the three FNII domains can bind to gelatin in isolation, although with variable affinity (Collier *et al.*, 1992). Again fragments containing all three tandem FNII domains show significantly stronger interaction than each one individually, indicating a cooperative binding activity (Banyai *et al.*, 1994). These observations raise the question as to how the single Endo180 FNII domain might mediate high-affinity collagen binding. Sequence analysis reveals

that the Endo180 FNII domain has conserved the N-terminal extension, which in the fibronectin ¹F2 domain functions to bring the N- and C- termini into close proximity (Pickford *et al.*, 1997, 2001), and single-particle electron microscopy studies have demonstrated that in Endo180 the FNII domain is intimately associated with adjacent cysteine-rich and CTLD1 domains (Rivera-Calzada *et al.*, 2003). This three-dimensional arrangement may function to stabilize the interaction with collagen. In support of a role of CTLD1 in maintaining the correct Endo180 confirmation, we find that Endo180-Fc constructs containing only the cysteine-rich and FNII domain are unstable. Furthermore, Endo180 is constitutively recruited into clathrin-coated pits on the cell surface (Isacke *et al.*, 1990), and therefore clustering of multiple Endo180 receptors may also enhance collagen binding.

Within its cytoplasmic domain, Endo180 contains a dihydrophobic endocytosis motif that is essential for the constitutive recruitment of this receptor into plasma membrane clathrin-coated pits. This is followed by rapid recruitment into the early endosomes and recycling back to the plasma membrane (Howard and Isacke, 2002). Such receptors typically internalize cargo that dissociates in the low pH endosomal environment and is subsequently targeted to the degradative compartments in the cell (Mellman, 1996; Gruenberg, 2001). Here we demonstrate that the constitutive endocytic property of Endo180 results in receptor:collagen complexes being rapidly internalized (<5 min) into early endosomes. As a consequence, cells expressing an Endo180 nonendocytic mutant are able to bind to, but not mediate uptake of, collagens. After delivery to the early endosomes, the collagenous cargo are seen to move quickly (<20 min) to an Endo180-negative compartment and by 1 h to be identified in LAMP1-positive degradative lysosomes. The absence of Endo180 colocalized with collagen in the later compartments indicates that the receptor is recycled ligand-free back to the plasma membrane.

In addition to collagens, Endo180 has been demonstrated to have two other binding activities. First, Endo180 can form a trimolecular cell surface complex with p α PA and uPAR (Behrendt *et al.*, 2000). The uPA/uPAR system is involved in a wide variety of vital cellular proteolytic and signaling functions ranging from cell adhesion and migration to angiogenesis and extracellular matrix remodeling (Preissner *et al.*, 2000; Mazar, 2001). Because uPAR is a glycosylphosphatidylinositol (GPI)-linked receptor, it requires associated receptors such as Endo180 to mediate intracellular signaling and/or regulate cell surface expression. The ability of collagen V to prevent formation of this trimolecular complex indicates a role for the Endo180 FNII domain in the interaction with uPA/uPAR and additionally suggests that there may be functional interplay between Endo180's role as a collagen receptor and a uPAR-associated protein. For example, it will be interesting to determine whether the interaction of Endo180 with uPA/uPAR leads to localized uPA-mediated activation of plasminogen and subsequent degradation of matrix components, including collagens, which could then be cleared from the microenvironment by Endo180-mediated endocytosis. Second, Endo180 is a functional C-type lectin showing Ca²⁺-dependent binding to mannose, fucose, and N-acetylglucosamine, and this binding typical of mannose-specific C-type lectin domains has been localized to Endo180 CTLD2 (East *et al.*, 2002). To date,

physiological glycosylated ligands have not been identified, but again the relatively close proximity of the FNII and CTLD2 domains may result in competitive ligand interactions.

Importantly, the studies described here demonstrate that Endo180 is not only a receptor for commercially obtained collagens as soluble Endo180 extracellular domain expressed in mammalian cells is able to interact with native collagens in tissue sections. This raises the questions as to what function Endo180 might have as a collagen receptor *in vivo*. Endo180 is an endocytic receptor and the immunofluorescence data presented here indicates that internalized collagens are taken up via the clathrin-coated pits into the early endosomes rather than by a phagocytic route. Consequently it would be anticipated that uptake of collagens would involve degraded fragments or unincorporated collagens rather than intact collagen fibrils, which would be too large for the size restricted clathrin-coated vesicles. Endo180 does not have intrinsic collagenase activity, suggesting it would act in concert with proteases in the remodeling of the matrix and/or to remove unwanted collagens before their incorporation into the fibrils, thereby allowing proper assembly of the matrix components. Although further studies will be required to understand the balance between Endo180's role as a binding vs. internalization receptor, it is of note that Endo180 is predominantly expressed on stromal fibroblasts and in sites of embryonic and neonatal chondrogenic and osteogenic activity (Isacke *et al.*, 1990; Wu *et al.*, 1996; Sheikh *et al.*, 2000; Engelholm *et al.*, 2001). This tissue distribution reflects cells involved in active matrix deposition and remodeling, and moreover, Endo180 is highly up-regulated on angiogenic compared with normal endothelial cells (St. Croix *et al.*, 2000) and on stromal fibroblasts and myoepithelial cells in breast tumors (Schnack Nielsen *et al.*, 2002), where increased collagen catabolism is required. Together these data implicate a physiological role for Endo180 in the regulation of the extracellular matrix, both in normal tissue maintenance and as a component of disease progression.

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NOTE ADDED IN PROOF

Since submission of this manuscript, two independent reports have demonstrated that fibroblasts from mice with a targeted Endo180 deletion show a collagen-binding defect (East *et al.*, 2003; Engelholm *et al.*, 2003).

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