Investigation of the role of Endo180/urokinase-type plasminogen activator receptor-associated protein as a collagenase 3 (matrix metalloproteinase 13) receptor

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Procollagenase 3 can be activated by interaction with and cleavage by the cell-associated membrane type 1 metalloproteinase (MT1 MMP; MMP 14). It has also been shown to bind to a specific receptor, and is subsequently internalized via the low-density lipoprotein-related receptor by osteoblast cell lines. The receptor was identified as a recycling glycoprotein of the macrophage mannose receptor family, Endo180. In order to ascertain whether there is a relationship between Endo180 binding and procollagenase 3 activation, we have compared procollagenase 3 activation by an HT1080 fibrosarcoma cell line overexpressing MT1 MMP, without and with overexpression of Endo180. No difference in procollagenase 3 activation was observed, and neither was the enzyme bound to the cells or

internalized. In contrast, the osteoblast cell lines, MG63 and UMR-106, both bound and internalized procollagenase 3. However, immunolocalization studies showed that the Endo180 abundantly expressed by these cells did not co-localize with the procollagenase 3. In further biochemical studies we confirmed that procollagenase 3 did not bind to Endo180, using both ligand-blotting and immunoprecipitation techniques. We conclude that Endo180 is unlikely to be a receptor for collagenase 3 in relation to either its activation or cell binding and internalization, and that other interaction partners must be sought.

Key words: MT1 MMP, MMP 13, uPA, uPAR.

INTRODUCTION

The activation of procollagenase 3 at the cell surface mainly occurs via one of two mechanisms. In the first, progelatinase A [pro-matrix metalloproteinase (MMP) 2] is activated by the membrane type 1 MMP (MT1 MMP) in a tissue inhibitor of metalloproteinases (TIMP)-2-dependent process, which then allows gelatinase A to activate procollagenase 3 [1,2]. In the second, activation of procollagenase 3 is directly mediated by MT1 MMP. Unlike the indirect mechanism, the latter is TIMP-2-independent and requires the presence of the intact C-terminal haemopexin-like domain of procollagenase 3 [1,3,4], suggesting that activation by the membrane-bound MT1 MMP may require the auspices of another procollagenase 3 cell surface receptor.

A specific cell surface receptor for rat collagenase 3 has been described on the osteosarcoma cell line UMR106-01 and is involved, in association with the low-density lipoprotein-related receptor (LRP) in the binding, internalization into lysosomes and subsequent degradation of collagenase 3 [5,6]. Subsequently it was demonstrated that this receptor has a broad cellular distribution, and sequencing of affinity-purified material revealed it to be the rat orthologue of Endo180 [7,8], a fourth member of the macrophage mannose receptor family [9]. Endo180 is a 180 kDa transmembrane glycoprotein and, like other members of the macrophage mannose receptor family, is comprised of a

large extracellular domain containing multiple C-type carbohydrate recognition domains. Both Endo180 and the macrophage mannose receptor are functional lectins, but with different sugar specificity; Endo180 binds to *N*-acetylglucosamine but not mannose [8]. A feature of this receptor family is the ability to internalize ligands, and with Endo180 it has been demonstrated that it is constitutively clustered into clathrin-coated pits on the plasma membrane, rapidly internalized into intracellular endosomes and then recycled back to the cell surface [7]. Endo180 has also been shown to be the cell surface protein binding the urokinase-type plasminogen activator receptor (uPAR) and has been named uPAR-associated protein (uPARAP) [10,11]. Most recently uPARAP mRNA was shown to be co-expressed with those of uPAR and collagenase 3 in osteoblast-type cells in the murine post-implantation embryo [12]. In the present study we have assessed the potential role of Endo180 in the binding, activation and internalization of procollagenase 3 using cell model systems.

EXPERIMENTAL

Cell lines

HTC75 is a human fibrosarcoma cell line (HT1080) into which the pTet off control element has been stably transfected, and these cells were further transfected with human MT1 MMP

Abbreviations used: DIG-NHS, digoxigenin-3-*O*-succinyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HRP, horseradish peroxidase; ICAM, intercellular cell-adhesion molecule; LRP, low-density lipoprotein-related receptor; MMP, matrix metalloproteinase; MT1 MMP, membrane type 1 MMP; TIMP, tissue inhibitor of metalloproteinases; uPAR, urokinase-type plasminogen activator receptor; uPARAP, uPAR-associated protein.

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cDNA in pTRE (ClonTech Laboratories) as described previously [13]. In these cells expression of MT1 MMP is repressed in the presence of 100 ng/ml doxycycline. HTC75 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 100 ng/ml doxycycline (Sigma) and 500 µg/ml Geneticin® (Gibco). Cells were induced to express MT1 MMP by culture in doxycycline-free DMEM for 24 h prior to experimentation. The rat osteosarcoma cell line UMR-106 (European Collection of Cell Cultures, Porton Down, Salisbury, Wilts., U.K.) and the human osteosarcoma cell line MG63 (provided by Dr I. Trowbridge; Salk Institute, San Diego, CA, U.S.A.) were maintained in DMEM containing 10% (v/v) FCS and antibiotics.

Western-blot analysis of cell lysates for LRP

MG63 cells were seeded into 6-well plates at a density of 2×10^5 cells/well in DMEM containing 10% (v/v) FCS, and were cultured for 72 h. HTC75 cells were seeded at 2×10^5 cells/well in DMEM containing doxycycline, and were cultured for 24 h. In transfection experiments, HTC75 cells were incubated with 2 µg of pcDNA3 Endo180 and 6 µl of FuGENE 6 (Roche) for 24 h. The cells were rinsed, the medium was replaced with fresh medium containing 10% (v/v) FCS and doxycycline, and the cells were left for a further 24 h. All cells were rinsed twice with Tris/saline [15 mM Tris/HCl (pH 7.5)/150 mM NaCl] and lysed in 60 µl of lysis buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 10 mM CaCl₂, 1 mM MgCl₂ and 0.15% Triton X-100] on ice for 15 min. Lysates were cleared by centrifugation and protein content was determined using the bicinchoninic acid ('BCA') assay (Sigma). Lysates were resolved on 5% (w/v) non-reducing polyacrylamide gels and transferred on to nitrocellulose membranes. LRP was detected with a monoclonal anti-LRP antibody followed by horseradish peroxidase (HRP)-conjugated anti-mouse Ig. Blots were developed using an enhanced chemiluminescent substrate (Pierce) and were exposed to X-ray film.

Activation experiments

HTC75 cells were seeded into 24-well plates at a density of 5×10^4 cells/well in DMEM containing doxycycline and 10% (v/v) FCS, and were cultured for 24 h. The medium was then changed to doxycycline-free medium and the cells were transfected overnight with 0.5 µg of pcDNA3 Endo180/well. Cells were subsequently rinsed twice with 1 ml of serum-free DMEM and were incubated with 100 ng of purified procollagenase 3 in 300 µl of serum-free DMEM for the time indicated in each experiment. The medium was removed and clarified by centrifugation, an aliquot was taken and proteins were separated on 10% (w/v) polyacrylamide gels and then transferred on to nitrocellulose membranes. Blots were probed with a sheep polyclonal anti-(collagenase 3) antibody (L29/6), which recognizes both the pro and active forms of the enzyme, followed by an HRP-conjugated donkey anti-sheep Ig.

Internalization experiments

HTC75, MG63 and UMR-106 cells were seeded into 24-well plates at a density of 5×10^4 cells/well. HTC75 cells were transfected with Endo180 as described for activation experiments, but were maintained in DMEM containing doxycycline. Cells were rinsed twice with 1 ml of serum-free DMEM and incubated with 100 ng of purified procollagenase 3 in 300 µl of serum-free DMEM for 24 h.

Digoxigenin labelling of procollagenase 3

Recombinant human procollagenase 3 was prepared as previously described [14], and labelled with digoxigenin using the digoxigenin antibody labelling kit supplied by Roche. Procollagenase 3 was dialysed overnight into Hepes buffer [50 mM Hepes (pH 7.6), 10 mM CaCl₂ and 150 mM NaCl], then incubated with a molar ratio of 1:10 procollagenase 3/digoxigenin-3-*O*-succinyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester (DIG-NHS) as recommended by the manufacturer. The labelling reaction was carried out for 6 h at 4 °C with gentle stirring. Remaining non-reacted DIG-NHS was separated by gel-filtration on a Sephadex G25 column equilibrated in Hepes buffer. Successful labelling was accompanied by an increase in the molecular size of procollagenase 3 separated by SDS/PAGE and visualized by silver staining. Alternatively, labelled procollagenase 3 was detected by Western-blot analysis using a mouse anti-digoxigenin HRP-conjugated antibody (Perbio Science UK Ltd, Tattenhall, Cheshire, U.K.) and a chemiluminescent substrate.

Immunofluorescence

For co-localization studies, cells on coverslips were rinsed twice and blocked at 4 °C for 30 min in medium containing 0.5% BSA. Cells were then incubated for 1 h at 4 °C with 1 µg/ml digoxigenin-labelled procollagenase 3 and either fixed and stained, or rinsed, incubated for a further 30 min at 37 °C to allow internalization of cell-surface bound procollagenase 3, and then fixed and stained. All cells were fixed in 3% (w/v) paraformaldehyde, and stained using rabbit anti-Endo180 polyclonal antibody followed by Alexa568 anti-rabbit Ig (Molecular Probes). In co-localization experiments procollagenase 3 was visualized using FITC-conjugated anti-digoxigenin F_{ab} fragments (Roche). As indicated, cells were permeabilized with 0.2% saponin.

Ligand blotting

An Endo180-F_c chimaera was generated by excising the complete Endo180 extracellular domain from the pcDNA3 Endo180 construct [8] using *Hind* III and ligating in frame into the pIgplus vector (R & D Systems). The intercellular cell-adhesion molecule (ICAM)-F_c chimaera in the pIgplus vector was provided by Dr D. Simmons (Celltech, Great Abington, Cambridge, U.K.). Chimaeras were expressed in COS cells and purified on Protein G columns. Each chimaera (15 ng) was resolved in duplicate on a non-reducing 7.5% (w/v) polyacrylamide gel and transferred on to nitrocellulose membrane. A concanavalin A dot-blot was prepared. The blots were blocked for 2 h at 20 °C with 5% (w/v) BSA in PBS. Blots were then incubated for 16 h in 5% (w/v) BSA/PBS with 20 nM digoxigenin-labelled procollagenase 3, followed by mouse anti-digoxigenin HRP-conjugated Ig (Pierce) to detect bound procollagenase 3. Alternatively, the chimaeras were detected using a goat anti-(human F_c) antibody followed by an HRP-conjugated anti-goat antibody.

Immunoprecipitation experiments

UMR-106 and MG63 cells were seeded into 10-cm dishes at a density of 4×10^5 cells in DMEM containing 10% (v/v) FCS, and were cultured for 72 h before lysis. HTC75 cells were seeded at a density of 2×10^5 cells in DMEM containing 10% (v/v) FCS and doxycycline, incubated for 24 h and transfected overnight

with 5 μ g of pcDNA3 Endo180 and 15 μ l of FuGENE 6. The cells were then rinsed, and the medium was replaced with fresh medium containing 10% (v/v) FCS and doxycycline for a further 24 h. For some experiments the cells were rinsed twice with DMEM containing 0.5% BSA, then incubated with 1 μ g/ml procollagenase 3 in DMEM containing 0.5% BSA for 1 h at 37 °C before lysis. The cells were rinsed twice with Tris/saline and lysed in 900 μ l of lysis buffer on ice for 15 min, the lysates were collected and cleared by centrifugation. To co-precipitate Endo180, Protein G–Sepharose (Sigma)-purified L29/6 sheep anti-(procollagenase 3) polyclonal antibody was used. Cell lysates were incubated for 1 h on ice with 25 μ g of preimmune serum, followed by 100 μ l of washed Protein G–Sepharose for 2 h at 4 °C. The lysates were centrifuged for 1 min and the supernatants collected. The supernatants were incubated with 100 ng of procollagenase 3 for 2 h at 4 °C. L29/6 (25 μ g) was added to each tube and incubated overnight at 4 °C. Washed Protein G–Sepharose (100 μ l) was added and incubated for 1 h at 4 °C. The lysates were spun down, the supernatants removed and the Protein G–Sepharose was then washed three times with 1 ml of lysis buffer. The Sepharose was boiled with 50 μ l of sample buffer, then spun down and the supernatant separated by SDS/PAGE (7 and 10% gels). The method used to co-precipitate procollagenase 3 using anti-Endo180 rabbit polyclonal serum was essentially the same. The lysates were pre-cleared with 25 μ g of rabbit preimmune serum, incubated with 1 μ g of digoxigenin-labelled procollagenase 3 for 2 h at 4 °C, and then incubated overnight with 0.5 μ l of anti-Endo180 rabbit polyclonal serum.

RESULTS

Transfection of MT1 MMP-expressing HTC75 cells with Endo180 cDNA has no effect on cellular activation of procollagenase 3

Procollagenase 3 is processed from an inactive 60 kDa form, via an intermediate, into its active 48 kDa form by cells expressing MT1 MMP on the cell surface [1]. A collagenase 3-specific lectin

Figure 2 Processing of procollagenase 3 by Endo180-transfected HTC75 cells

MT1 MMP-expressing HTC75 cells either transfected (+) or not transfected (–) with Endo180 cDNA were incubated with 100 ng of procollagenase 3 for 0–8 h at 37 °C. Medium was removed and analysed by Western blotting using anti-(collagenase 3) antibody L29/6, which recognizes both the pro and active forms of the enzyme.

receptor was shown to be present on the surface of the rat osteosarcoma cell line UMR106-01 [5]. In view of the high affinity of this specific receptor for procollagenase 3 ($K_d = 5 \times 10^{-9}$ M) [15], we wished to investigate the effect of Endo180, the human orthologue of this receptor, on the activation of procollagenase 3 by MT1 MMP. For this purpose, HTC75 cells were used in which expression of MT1 MMP can be induced by culture in doxycycline-free medium [13,16]. These cells were transiently transfected with Endo180, and expression of the protein was analysed by both Western blotting (results not shown) and immunofluorescence. Figure 1 shows that HTC75 cells express endogenous Endo180 (Figure 1a), and that this expression is dramatically increased by transfection of the cells with the cDNA (Figure 1b). In parallel, UMR-106 cells (Figures 1c and 1d) and MG63 cells (Figures 1e and 1f) were also stained. MG63 is a human osteosarcoma cell line which has been shown to express high levels of Endo180 (approx. 10^6 molecules/cell) [7]. The distribution of endogenous and transfected Endo180 is as expected in all three cell types [7,8]. In non-permeabilized cells, a punctate staining is observed on the cell surface, indicative of clustering into clathrin-coated pits, whereas in permeabilized cells a strong endosomal staining is present.

The effect of transfection with Endo180 on the activation of procollagenase 3 by HTC75 cells induced to express MT1 MMP by culture in doxycycline-free medium was assessed. Over an 8 h time period there is activation of procollagenase 3 by the non-transfected cells (Figure 2), a process which is complete by 24 h (results not shown). Transfection of the cells with Endo180 has no effect on the rate of activation of procollagenase 3 by the cells over 8 h, and again activation is complete after 24 h. Transfection of HTC75 cells induced to express MT1 MMP with Endo180 had no effect on levels of expression of MT1 MMP by the cells (results not shown).

HTC75 cells transfected with Endo180 cDNA do not internalize procollagenase 3

Endo180 is an endocytic recycling glycoprotein [7]. In order to establish whether Endo180 is involved in the uptake of procollagenase 3 from the medium, both Endo180-transfected and non-transfected HTC75 cells, and UMR-106 and MG63 cells were incubated with 100 ng of procollagenase 3 for 24 h and the medium was collected and analysed by Western blotting (Figure 3a). Following a 24 h incubation period, the concentrations of procollagenase 3 present in the medium collected from both non-transfected (lanes 1 and 2) and Endo180-transfected HTC75 cells (lanes 3 and 4) are equal to each other and to the original concentration of procollagenase 3 added to the cells (lane 9). By contrast, both the UMR-106 (lanes 5 and 6) and the MG63 (lanes 7 and 8) cells have internalized nearly all of the pro-

Figure 1 Expression of Endo180 by HTC75, UMR-106 and MG63 cells

HTC75 cells either not transfected (a) or transfected with human Endo180 (b), UMR-106 cells (c and d) and MG63 cells (e and f) were fixed and stained with an anti-Endo180 antibody followed by Alexa568-conjugated anti-rabbit Ig in the absence (c and e) or presence (a, b, d and f) of 0.2% saponin. For (a and b) the bar in (a) = 100 μ m, and for (c–f) the bar in (c) = 25 μ m.

Figure 3 UMR-106 and MG63 cells internalize procollagenase 3

(a) HTC75 cells cultured in the presence of doxycycline and either not transfected (lanes 1 and 2) or transfected with Endo180 (lanes 3 and 4), UMR-106 cells (lanes 5 and 6) and MG63 cells (lanes 7 and 8) were incubated with 100 ng of procollagenase 3 for 24 h at 37 °C, the conditioned medium from duplicate wells was removed and procollagenase 3 content compared with a control (lane 9) by Western-blot analysis, using antibody L29/6. (b) Endo180-transfected HTC75 cells (lane 1) and MG63 cells (lane 2) were analysed for LRP expression by Western blotting of cell lysates.

collagenase 3 added to the medium. In previous studies, internalization of collagenase 3 by the specific receptor identified on UMR106-01 cells [6] was shown to require LRP. We used Western blotting to demonstrate that LRP is present in both Endo180-transfected HTC75 cells (Figure 3b, lane 1) and MG63 cells (Figure 3b, lane 2).

MG63 and UMR-106 cells bind digoxigenin-labelled procollagenase 3, whereas both Endo180-transfected and non-transfected HTC75 cells do not

Transfection of HTC75 cells with Endo180 had no effect on the rate of activation of procollagenase 3 by cells expressing MT1 MMP. Removal of procollagenase 3 from the conditioned medium by Endo180-transfected cells could not be detected by Western-blot analysis. Therefore it was necessary to determine whether there is indeed any evidence of an interaction between Endo180 and procollagenase 3. To address this, the binding of procollagenase 3 to cultured cells was first assessed by immunofluorescence. To increase the sensitivity of the assay, cells were incubated with digoxigenin-labelled procollagenase 3 followed by an FITC-conjugated anti-digoxigenin secondary antibody. UMR-106 and MG63 cells were incubated with procollagenase 3 at 4 °C for 1 h. The cells were then either fixed and surface stained (Figures 4a and 4b), or were rinsed with medium and incubated for a further 30 min at 37 °C, to allow internalization of surface-bound procollagenase 3. The cells were then either surface stained (Figures 4c and 4d) or permeabilized with 0.2 % saponin and stained (Figures 4e and 4f).

Procollagenase 3 was clearly bound to the surface of both UMR-106 (Figure 4a) and MG63 cells (Figure 4b) after incubation at 4 °C. Incubation at this temperature allows binding of procollagenase 3 to the cell surface, but prevents both clustering and internalization. A punctate distribution of procollagenase 3 is observed on the cell surface, but this does not coincide with Endo180 staining. A similar punctate pattern of staining is also detected on the surface of UMR-106 (Figure 4c) and MG63 cells (Figure 4d) after warming to 37 °C for 30 min. Warming will allow clustering and internalization of any cell-surface-bound procollagenase 3. Some clustering of procollagenase 3 may be present on the cell surface, but this is not accompanied by clustering of Endo180. The typical internal pattern of Endo180 staining is seen in permeabilized cells (Figures 4e and 4f). Here there is some evidence of procollagenase 3 internalization, but

Figure 4 Procollagenase 3 bound to UMR-106 and MG63 cells does not co-localize with Endo180

UMR-106 (a, c and e) and MG63 (b, d and f) cells were incubated at 4 °C with digoxigenin-labelled procollagenase 3. The cells were then either fixed and stained with anti-Endo180 serum followed by Alexa568 anti-rabbit Ig (red) and FITC-conjugated anti-digoxigenin Ig (green) (a and b), or incubated at 37 °C for 30 min and then fixed and stained, in either the absence (c and d) or presence (e and f) of 0.2% saponin to permeabilize the cells. Non-transfected (g) and Endo180-transfected (h) HTC75 cells were incubated with procollagenase 3 at 37 °C, fixed and stained with FITC-conjugated anti-digoxigenin. Bar = 25 µm.

Figure 5 Procollagenase 3 does not bind to purified Endo180, as determined by ligand blotting

Endo180-Fc and ICAM-Fc chimaeras were resolved by SDS/PAGE in duplicate under non-reducing conditions and were transferred on to nitrocellulose. (a) Probed with HRP-conjugated anti-Fc. The presence of both monomeric and dimeric chimaeras can be seen. (b) Incubated overnight with 20 nM digoxigenin-labelled procollagenase 3 followed by HRP-conjugated anti-digoxigenin antibody. (c) A concanavalin A (Con A) dot-blot was processed in parallel with the ligand blot (b) as a positive control for procollagenase 3 binding.

again this does not coincide with Endo180 staining. No procollagenase 3 binding to the surface of either transfected (Figure 4g) or non-transfected (Figure 4h) HTC75 cells was detected, and, consistent with the data shown in Figure 3, there was no evidence of procollagenase 3 internalization after incubation at 37 °C (results not shown).

Binding of digoxigenin-labelled procollagenase 3 to Endo180 cannot be detected using ligand blotting techniques

Ligand blotting of UMR106-01 cell membranes was one of the techniques used to detect the 170 kDa specific receptor for collagenase 3 on UMR106-01 cells [6]. Consequently, we used ligand blotting to determine whether procollagenase 3 could bind to a purified Endo180 preparation (Figure 5). An Endo180-Fc chimaera together with an ICAM-Fc chimaera as a negative control were resolved on a non-reducing SDS/polyacrylamide gel and transferred on to nitrocellulose membranes. To confirm loading, Endo180-Fc and ICAM-Fc chimaeras were detected using an anti-Fc antibody (Figure 5a). A parallel blot was incubated with digoxigenin-labelled procollagenase 3 followed by an HRP-conjugated anti-digoxigenin antibody (Figure 5b). As a positive control, dot-blot of the lectin concanavalin A, which is known to bind procollagenase 3, were included in the assay (Figure 5c). Binding of digoxigenin-labelled procollagenase 3 to the concanavalin A dot-blot was readily detectable using this method. However, no binding of procollagenase 3 to Endo180 is seen even after overnight exposure of the blots to X-ray film.

Endo180 and procollagenase 3 do not co-immunoprecipitate

As a final approach to detect any interaction between Endo180 and collagenase 3, co-immunoprecipitation experiments were

Figure 6 Endo180 and procollagenase 3 do not co-precipitate

(a and b) Cell lysates preincubated either without (—) or with (+) digoxigenin-labelled procollagenase 3 were immunoprecipitated with anti-Endo180 serum and Protein G-Sepharose. Immunoprecipitates were resolved by SDS/PAGE, transferred on to nitrocellulose and probed with anti-Endo180 serum (a) or HRP-conjugated anti-digoxigenin Ig (b). (c and d) Cell lysates preincubated without (—) or with (+) procollagenase 3 were immunoprecipitated with anti-collagenase 3 antibody followed by Protein G-Sepharose. Immunoprecipitates were resolved by SDS/PAGE, transferred on to nitrocellulose and probed with anti-collagenase 3 (c) or anti-Endo180 serum (d). Arrowheads indicate the expected positions of digoxigenin-labelled procollagenase 3 (b) and Endo180 (d).

undertaken using MG63 and UMR-106 cells, and Endo180-transfected and non-transfected HTC75 cells (Figure 6). Cells were lysed, incubated with or without procollagenase 3 at 4 °C and then immunoprecipitated with anti-Endo180 polyclonal antibody. In these experiments, Endo180 was successfully immunoprecipitated from the cell lysates (Figure 6a), however, no co-immunoprecipitation of digoxigenin-labelled procollagenase 3 was detected (Figure 6b) even from MG63 and UMR-106 cells to which collagenase 3 has been shown to bind (Figure 4). Similarly, no co-immunoprecipitation was observed after cells had been incubated with unlabelled procollagenase 3 for 1 h at 37 °C prior to lysis (results not shown). Reciprocal experiments were also performed in which cells were incubated for 1 h at 37 °C with unlabelled procollagenase 3, lysed, immunoprecipitated with an anti-(collagenase 3) antibody and the immunoprecipitates blotted for Endo180. Collagenase 3 was successfully immunoprecipitated from the lysates (Figure 6c) but no co-immunoprecipitating Endo180 was detected (Figure 6d).

DISCUSSION

In earlier studies we have shown that recombinant procollagenase 3 or a pro form of the catalytic domain alone could be activated directly by the soluble form of MT1 MMP and that activation was potentiated by gelatinase A [1]. However, membrane-bound MT1 MMP, in the form of cell lysates, or intact cells expressing MT1 MMP, could only activate the full-length procollagenase 3. Based on this observation that procollagenase 3 could only be activated by cell surface MT1 MMP if the haemopexin-like

domain was present, we postulated a potential interaction with an accessory binding protein on cell membranes. The discovery of a specific collagenase 3 receptor and its identification as the orthologue of human Endo180/uPARAP [6,8,10] prompted us to investigate the possibility that Endo180 might act as such a binding protein and function to potentiate activation of procollagenase 3. However, when Endo180 was overexpressed in the HTC75 cell line in the presence of high levels of MT1 MMP, and hence procollagenase 3-activating capacity, no change in procollagenase 3 activation rates was observed. Furthermore, these cells did not have an enhanced capacity to internalize and degrade procollagenase 3. Given these results we undertook a number of experiments to determine directly whether Endo180 could bind collagenase 3. As described elsewhere [5,6], Endo180 is found on the cell surface clustered into clathrin-coated pits and intracellularly in endosomes. In all the cells lines employed in the present study, a similar distribution of both endogenous and transfected Endo180 was observed. However, digoxigenin-labelled procollagenase 3 did not bind at all to HTC75 cells, despite high levels of expression in the Endo180-transfected cells. However, these cells are able to efficiently activate the proenzyme as long as the haemopexin domain is present, which had been interpreted to imply some form of interaction. Binding was observed to the UMR-106 and MG63 cells but, in this case, bound procollagenase 3 did not co-localize with endogenous Endo180. Although approx. 90% of the collagenase 3 preparation was in the pro form, we noted that a complex of active collagenase 3 with TIMP-1 also did not associate with Endo180 (results not shown). The lack of binding of procollagenase 3 to Endo180 was confirmed using both ligand blotting and co-immunoprecipitation techniques. Hence, although collagenase 3 may be expressed at the same time as Endo180 in cells of the osteoblast lineage during primary ossification of developing mouse long bones [12], we conclude that they are unlikely to be involved in direct interactions. Collagenase 3 is a highly glycosylated protein and binds to lectins, such as concanavalin A, but it seems that the specific receptor found on bone and other mesenchymal cells is not the C-type lectin Endo180 and its exact identity remains to be elucidated.

Despite the lack of evidence described in the present study for Endo180 being the specific collagenase 3 receptor, it remains a strong possibility that this recycling receptor does play a role in regulating protease activity at the cell surface. Endo180 is also referred to as uPARAP due to the demonstration in cross-linking studies that it forms a complex on the cell surface with pro-uPA-bound uPAR [10]. Interestingly, this interaction can be inhibited by collagen V and it is likely that this is mediated by the fibronectin type II domain in Endo180/uPARAP as this domain has been demonstrated in other proteins to mediate interaction with collagens. Endo180/uPARAP recycles between the clathrin-coated pits on the cell surface and intracellular endosomes. Receptors which show this type of trafficking typically release bound ligand in the low-pH endosomal environment. Together with the demonstration that Endo180 has calcium-dependent lectin activity [8], these data have led to the suggestion that Endo180/uPARAP may have a function in controlling the level of extracellular uPA/uPAR and/or the uptake and degradation of extracellular matrix components and glycopro-

teins [11]. Certainly Endo180/uPARAP is a multidomain protein which can bind multiple ligands, but we conclude that collagenase-3 is not one of these ligands.

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