uPARAP/Endo180 is essential for cellular uptake of collagen and promotes fibroblast collagen adhesion

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The uptake and lysosomal degradation of collagen by fibroblasts constitute a major pathway in the turnover of connective tissue. However, the molecular mechanisms governing this pathway are poorly understood. Here, we show that the urokinase plasminogen activator receptor–associated protein (uPARAP)/Endo180, a novel mesenchymally expressed member of the macrophage mannose receptor family of endocytic receptors, is a key player in this process. Fibroblasts from mice with a targeted deletion in the uPARAP/Endo180 gene displayed a near to complete abrogation of collagen endocytosis. Furthermore, these cells had diminished initial adhesion to a range of different collagens, as well as impaired migration on fibrillar collagen. These studies identify a central function of uPARAP/Endo180 in cellular collagen interactions.

Introduction

The capacity of multicellular organisms to remodel the ECM is essential for development, homeostasis, and postnatal tissue remodeling, but also for the progression of many degenerative and proliferative diseases, including cancer. Both physiological and pathological matrix remodeling depend on an intricate interplay between cell motility factors, cell matrix adhesion receptors, and cell surface–associated proteases (Murphy and Gavrilovic, 1999). The interstitial collagens are the most abundant protein constituents of connective tissues and they, like other matrix components, are undergoing continuous synthesis and degradation. The pathway proposed to be the major clearance mechanism for collagen under steady-state conditions involves the specific binding of collagen fibrils to the cell surface, followed by the cellular uptake and degradation of the internalized collagen in the lysosomal compartment (Everts et al., 1996). However, unlike the alternative turnover mechanisms that are dominated by extracellular collagen degradation, this pathway is still poorly understood. It is believed to be primarily operative in fibroblasts, and results in the degradation of denatured collagen by proteases within the acidified lysosomal environment (Kiely et al., 1993; Lee et al., 1996; Segal et al., 2001). However, the molecular mechanisms behind the cellular internalization of the collagen substrate are largely unknown.

The urokinase plasminogen activator receptor–associated protein (uPARAP)/Endo180 is a novel multi-domain transmembrane glycoprotein that was identified through its specific interaction with receptor-bound pro-urokinase plasminogen activator on the surface of certain cultured cells (Behrendt et al., 2000) and, independently, as a constitutive endocytic glycoprotein (Howard and Isacke, 2002; Sheikh et al., 2000). uPARAP/Endo180 is a member of the macrophage mannose receptor family of type I transmembrane glycoproteins (Engelholm et al., 2001a). The distinct and highly conserved domain structure that characterizes this protein family includes an NH2-terminal, cysteine-rich ricin B lectin-like domain, a fibronectin type II (FN-II) domain, a series of 8–10 domains related to C-type carbohydrate rec-
ognition domains, a transmembrane domain, and a short COOH-terminal cytoplasmic tail (Sonnhammer et al., 1998; Bateman et al., 1999; Engelholm et al., 2001a). uPARAP/Endo180 is highly expressed on osteoblasts and osteocytes at sites of endochondral and intramembranous ossification during development (Engelholm et al., 2001b). The postnatal expression of uPARAP/Endo180 is restricted to specific subsets of fibroblasts, macrophages, and endothelial cells (Sheikh et al., 2000). The biochemical functions of uPARAP/Endo180 have yet to be elucidated. However, several conspicuous properties of the receptor suggest that uPARAP/Endo180 may be involved in the remodeling of the ECM and/or modulation of the localization or availability of soluble ligands in the pericellular environment (Hanasaki and Arita, 1999; Martinez-Pomares et al., 1999; Sheikh et al., 2000; Engelholm et al., 2001a). Of particular interest, initial studies on uPARAP/Endo180 revealed that the uPAR-dependent binding of pro-urokinase plasminogen activator to this receptor is blocked efficiently by low concentrations of collagen type V, suggesting a direct interaction with this constituent of the ECM. This function is likely to be mediated by the FN-II domain because this domain type is typically associated with collagen binding (Ancian et al., 1995). In this work, we generated a targeted deletion in the uPARAP/Endo180 gene and show that uPARAP/Endo180 is essential for the uptake of collagen by fibroblasts, and that it has important functions in fibroblast adhesion and migration on fibrillar collagen matrices.

Results and discussion
Targeted inactivation of the uPARAP/Endo180 gene in mice
We targeted the uPARAP/Endo180 gene in mice by replacing exons 2–6 with an HPRT expression cassette (Fig. 1 A). The correct targeting of the uPARAP/Endo180 gene was con-
firmed by Southern hybridization (Fig. 1 B) and by the presence of a truncated uPARAP/Endo180 transcript in mice homozygous for the insertion (uPARAP/Endo180<sup>−/−</sup> mice), as determined by Northern blot hybridization (Fig. 1 C). Western blot analysis of extracts of cultured fibroblasts from uPARAP/Endo180<sup>−/−</sup> mice, using an antibody directed against an epitope (residue 809–829) located outside of the introduced deletion, confirmed the absence of intact uPARAP/Endo180 (Fig. 1 D). A weak band with an apparent M<sub>r</sub> of 70 kD was observed in Western blots of homozygously targeted as well as wild-type and heterozygous mice (unpublished data). This product was seen in all genotypes, suggesting that it was due to a weak cross-reactivity of the antibody rather than a putative truncated uPARAP/Endo180 gene product. However, it should be noted that even though formation of a truncated uPARAP/Endo180 in the targeted mice could not be formally excluded, any such product would be devoid of the FN-II domain due to the targeting strategy (Fig. 1 A, legend).

**uPARAP/Endo180 is required for collagen internalization**

Dermal fibroblasts abundantly express uPARAP/Endo180 (Engelholm et al., 2001b). Therefore, we generated matched pairs of uPARAP/Endo180<sup>−/−</sup> and uPARAP/Endo180<sup>+/+</sup> littermate control fibroblasts, providing an ideal tool for studying the function of uPARAP/Endo180 in cell matrix interactions and the association of uPARAP/Endo180 with extracellular ligands. We investigated if uPARAP/Endo180 serves directly as an internalization receptor for collagen. The capacity of uPARAP/Endo180<sup>−/−</sup> fibroblasts to internalize <sup>125</sup>I-labeled collagen was determined and compared with the internalization of <sup>125</sup>I-labeled holotransferrin, matrix metalloproteinase (MMP)-13, and fibronectin as independent controls.

Strikingly, the uPARAP/Endo180-deficient fibroblasts displayed a virtually complete abrogation of the cellular uptake of collagen types I, IV, and V (Fig. 2 A, panels 3, 4, and 1, respectively). This deficiency in collagen internalization was observed with several independent isolates of uPARAP/Endo180<sup>−/−</sup> fibroblasts and could be alleviated by the reintroduction of uPARAP/Endo180 by transient transfection of the cells with a uPARAP/Endo180 cDNA expression vector (Fig. 2 A, panel 2; unpublished data). In contrast, the cellular uptake of holotransferrin, MMP-13, and fibronectin was unaffected (Fig. 2 A, panels 5–7). The internalization of MMP-13 has been shown previously to be uPARAP/Endo180-independent (Bailey et al., 2002), but depends on the low density lipoprotein receptor–related protein (LRP; Barmina et al., 1999). Therefore, as an additional test of our assay system, we included samples with LRP-deficient cells.
The percentage of adherent uPARAP/Endo180 of cells adhering to the different matrices was type IV (C), type V (D), total tendon collagen (E), and E, P in this series of events.

Lar internalization of collagen thus addresses a central step in these events. Everts and Beertsen, 1992). Our current demonstration of this process of collagen by lysosomal proteases (Everts et al., 1988, 1996; Everts et al., 1989). The major degradation process can occur in the absence, or at least with only very low levels, of accessible integrin on the cell surface.

The cellular uptake of collagen has been reported to be insensitive to inhibitors of MMPs, serine proteases, and cysteine proteases, and extensive degradation of collagen before the internalization step does not seem to be needed (Everts et al., 1988, 1989). The major degradation processes in this pathway occur after the cellular uptake event, by the fusion of collagen-containing intracellular vesicles with lysosomes and the degradation of acid-denatured collagen by lysosomal proteases (Everts et al., 1988, 1996; Everts and Beertsen, 1992). Our current demonstration of a crucial function of uPARAP/Endo180 in the actual cellular internalization of collagen thus addresses a central step in this series of events.

uPARAP/Endo180-deficient cells display delayed adhesion to collagen matrices

We used a standard cell adhesion assay, in which cells are allowed to attach briefly to immobilized collagens, to investigate if the lack of uPARAP/Endo180 could directly influence cell adhesion. Loss of uPARAP/Endo180 resulted in a 50% reduction in the adhesion of fibroblasts to type V collagen-coated surfaces (Fig. 3 D). This impairment was observed in comparisons of several independent isolates of uPARAP/Endo180−/− fibroblasts with littermate control fibroblasts. Interestingly, uPARAP/Endo180−/− cells also demonstrated a reduced ability to adhere to other immobilized collagens, including purified types I and IV collagen and total tendon collagen (Fig. 3, A, C, and E), although the reduction in these cases was less dramatic. In contrast, uPARAP/Endo180 deficiency did not affect the adhesion of fibroblasts to a noncollagen substrate such as fibronectin (Fig. S2 C) or a surface coated with total serum proteins (Fig. 3 F). A time-course study of cell adhesion to collagen V and collagen I showed that the effect of uPARAP/Endo180 deficiency was limited to the initial phase (15–30 min after plating), and wild-type and targeted cells reached the same level of adhesion after 1–2 h (unpublished data).

Because α1β1 integrin is considered important in various cellular interactions with collagen as stated in the previous section, it was important to determine if the role of uPARAP/Endo180 in these adhesion processes was dependent on additional integrin-mediated interactions. Therefore, we performed a new series of adhesion experiments in the presence of the same blocking antibody against β1 as used above in the internalization studies, or an irrelevant control antibody (anti-TNP). This experiment (Fig. S2) confirmed the central function of β1 in fibroblast adhesion to collagen, as well as to fibronectin used as a positive control. Adhesion to collagen I, collagen V, and fibronectin-coated surfaces was reduced to levels close to baseline by the anti-β1 antibody, irrespective of whether uPARAP/Endo180−/− or uPARAP/Endo180+/+ fibroblasts were used. The effect of uPARAP/Endo180 deficiency on collagen adhesion observed in the absence of anti-β1 antibody, however, was not due to a down-regulation of this integrin on the targeted cells. Thus, flow cytometry analysis (Fig. S3) showed that β1 expression levels on wild-type and uPARAP−/− cells were identical or closely similar. The two cell types also had indistinguishable morphologies after plating on various matrices (Fig. S4).
These experiments revealed that uPARAP/Endo180 has an early modulatory function in fibroblast adhesion to collagen matrices, whereas β1 integrins appear to be indispensable for this process. Thus, our observations make it tempting to speculate that uPARAP/Endo180 is critical for fully effective initial cellular interactions with collagen, this role having an impact on collagen adhesion, beyond its crucial role in collagen internalization. A simple cell binding assay with solubilized $^{125}$I-labeled collagen V at 4°C supported this notion, as a >50% reduction in binding was observed with uPARAP/Endo180$^{-/-}$ cells (unpublished data).

**uPARAP/Endo180 deficiency impairs the migration of fibroblasts on collagen fibrils**

Cell migration is intimately linked with adhesion to the ECM (Murphy and Gavrilovic, 1999). To directly test if uPARAP is important for cellular migration on collagen, we performed single-cell, parallel time-lapse video microscopy of matched pairs of primary dermal uPARAP/Endo180$^{-/-}$ and littermate control fibroblasts on a mixed fibrillar collagen matrix (Fig. 4, A and B). uPARAP/Endo180$^{-/-}$ cells demonstrated a significant and reproducible impairment in their migration, displaying a >30% reduction in the average migration rate. The mechanistic details of this effect await further studies. One role of uPARAP/Endo180 in cell migration may be directly related to increasing cellular adhesion. However, uPARAP/Endo180-mediated collagen internalization may also promote migration by increasing adhesion site turnover, thereby facilitating cell spreading. Finally, the interplay between uPARAP/Endo180 and integrins may influence integrin-mediated signaling.

In summary, we have shown that uPARAP/Endo180, a mesenchymal cell surface receptor, has a pivotal function in collagen internalization, facilitates the initial adhesion of fibroblasts to collagen, and accelerates the migration of fibroblasts on a fibrillar collagen matrix. The fact that uPARAP/Endo180-targeted mice do not present an obvious, aberrant phenotype makes it clear that uPARAP/Endo180 deficiency is tolerable in the absence of external challenges. Several pathways for collagen turnover operate in parallel (Holmbeck et al., 1999), and apparently these mechanisms can functionally compensate for the loss of uPARAP/Endo180 to an extent that prevents a robust presentation of a phenotype. The generation of mice with combined deficiencies in several collagen degradation pathways may provide valuable information as to the interplay and functional overlap between the pericellular and endocytic pathways of collagen degradation, and may shed more light on the specific functions of uPARAP/Endo180 in vivo.

**Materials and methods**

**uPARAP/Endo180 gene targeting**

uPARAP/Endo180 gene targeting was accomplished by replacement of exons 2–6 with an HPRT cassette in embryonic stem cells, followed by the generation of chimeric mice and interbreeding of chimeric offspring carrying fibroblasts selected randomly. The cells were tracked for 6 h by time-lapse microscopy. Size bar is indicated in the lower right corner of the control cell panel.
ing the disrupted uPARAP/Endo180 allele (Fig. 1A). uPARAP/Endo180−/− mice were derived from two independently targeted embryonic stem cell clones (see supplemental materials and methods for further details).

**Western blot analysis**

Synthesis of peptide 809–829 of the murine uPARAP/Endo180 sequence (GenBank/EMBL/DDB accession no. AAC52729), immunization of rabbits, affinity purification of antibodies and Western blotting of cell lysates were performed as described previously (Schnack Nielsen et al., 2002) using the antibody at a final concentration of 2 μg/ml.

**Ligand internalization assay**

Primary skin fibroblasts were isolated from neonates as described previously (Holmbeck et al., 1999). 20 μg type I collagen, type IV collagen, type V collagen, human brain transferrin (all from Calbiochem-Novacem), and human plasma fibronectin (Sigma-Aldrich) were labeled with 125I as described previously (Behrendt et al., 1996). Cellular ligand internalization assays were performed as described previously (Hahn-Dan
tona et al., 2001). In brief, samples of 105 cells were seeded in 24-well tissue culture plates covered with rat tail tendon collagen as described above. The dishes were coated with rat tail tendon collagen (provided by Dr. Jack Windsor, University of Indianapolis, Indianapolis, IN), human type I collagen, murine type II collagen, and human type IV collagen (all from Calbiochem-Novacem), at 20 μg/ml in 10 mM acetic acid. The coated wells were washed three times with PBS, and the residual binding sites were blocked by incubation with 0.2% BSA in PBS for 1 h at 37°C, followed by three additional washes with PBS at RT. Primary fibroblasts were detached by mild trypsinization, washed, resuspended in serum-free medium with 1% Nutridoma-SP (serum substitute; Roche). The medium was removed and replaced by binding buffer with 125I-labeled protein ranging in concentration from 0.5 to 5 nM, followed by incubation at 37°C. In some experiments, the cells were preincubated for 30 min at 4°C with 10 μg/ml anti-β1 antibody (HA2/5) or 10 μg/ml isotype-matched control anti-Thy-1 antibody (G215–1; both from BD Biosciences) before the addition of labeled ligand. The cells were then washed twice with ice-cold PBS, and incubated <2 min at 4°C with 50 μg/ml trypsin, 50 μg/ml proteinase K, and 0.53 mM EDTA in HBSS. The detached cells were centrifuged at 3,000 rpm for 5 min at 4°C, and the radioactivity in the pellet (internalized material) and supernatant (surface-released material) was measured in a gamma counter. Statistical significance was calculated by two-tailed t test.

**Transient transfection of primary fibroblasts**

Transfection of cultured fibroblasts was performed essentially as described previously (Kjelller and Hall, 2001), using the vector pcdNA3-uPARAP/Endo180 (provided by Dr. Clare Isacke, Chester Beauty Laboratories, London, UK; Sheikh et al., 2000). The transfection efficiency, estimated by determination of the percentage of GFP-expressing cells after cotransfection with pEGF-P1 (CLONTECH Laboratories, Inc.) was ~10%.

**Cell adhesion assay**

96-well plates were coated for 1 h at 37°C with either 10% FCS in DME, or with rat tail tendon collagen (provided by Dr. Jack Windsor, University of Indianapolis, Indianapolis, IN), human type I collagen, murine type II collagen, human type IV collagen, or human type V collagen (all from Calbiochem-Novacem-Novacem), at 20 μg/ml in 10 mM acetic acid. The coated wells were washed three times with PBS, and the residual binding sites were blocked by incubation with 0.2% BSA in PBS for 1 h at 37°C, followed by three additional washes with PBS at RT. Primary fibroblasts were detached by mild trypsinization, washed, resuspended in serum-free medium with 0.2% BSA, and incubated at 37°C for 30 min with gentle rotation to recover from trypsinization. Cell viability was determined by Trypan blue exclusion. 5 × 104 cells were added to each well and were allowed to attach for 30 min at 37°C. Nonadherent cells were removed by two gentle washes with 200 μl PBS, and the number of adherent cells was determined by MTT analysis (Liu et al., 2001). In some experiments, cells were preincubated with anti-β1 antibody or control antibody as specified above. Statistical significance was calculated by two-tailed t test.

**Cell migration assay**

Tissue culture plates with a glass coverslip bottom (MatTek Corporation) were coated with rat tail tendon collagen as described above. The dishes were washed twice with PBS, and incubated for 1 h at 37°C with 2% heat-denatured BSA in PBS to block residual binding sites. The integrity of the collagen fibrils was verified by incubation with purified trypsin and MMP-9 (Netzel-Arnett et al., 2002). uPARAP/Endo180−/− or uPARAP/Endo180-expressing uPARAP/Endo180−/− littermate control fibroblasts (1,000 cells/well) were seeded overnight on the collagen layer in DME containing 10% FCS. FCS was included during seeding and microscopy due to the long duration of the experiments. Cell migration was analyzed by time-lapse video microscopy as described previously (Gu et al., 1999). In brief, cell movements were recorded using inverted microscopes (Carl Zeiss MicroImaging, Inc.), collecting video images with tube cameras (Newvicon model 2400; Hamamatsu Photonics) at 10-min intervals for at least 6 h. From the individual cell tracks, cell velocities were calculated using MetaMorph® 4.6 software (Universal Imaging Corporation). A one-way ANOVA and a Bonferroni multiple comparisons post-test were performed using InStat® software (GraphPad Software, Inc.) to determine the statistical significance between samples. The experiment was repeated with four independently isolated pairs of uPARAP/Endo180−/− and littermate-matched uPARAP/Endo180−/− fibroblasts, and included a total of 52 and 82 individual measurements, respectively.

**Flow cytometry**

Cells were detached in PBS with 5 mM EDTA, 5 mg/ml BSA, washed twice in binding buffer (PBS with 0.1% mg/ml CaCl2, 0.05% mg/ml MgCl2, 1 mg/ml NaN3, and 5 mg/ml BSA), and incubated for 30 min at 4°C with either FITC-conjugated anti-p1 integrin antibody (HA2/5) or FITC-conjugated iso
type-matched control anti-TNP antibody (G235–1), using 5 μg of antibody for 106 cells in 200 μl binding buffer. After incubation, cells were washed twice in ice-cold binding buffer and fixed in 1% PFA in binding buffer without NaN3. Flow cytometry analysis was then performed using a FACs
tm (Becton Dickinson).

**Online supplemental material**

Supplemental materials and methods, and additional primary data are available at (http://www.jcb.org/cgi/content/full/jcb.200211091/DC1).

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