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 (Kielty 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 recycling glycoprotein (Endo180) that is capable of internalizing mAbs directed against it (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 NH$_2$-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-
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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 typically associates 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. 1A). The correct targeting of the uPARAP/Endo180 gene was con-
confirmed by Southern hybridization (Fig. 1 B) and by the presence of a truncated uPARAP/Endo180 transcript in mice homozygous for the insertion (uPARAP/Endo180−/− mice), as determined by Northern blot hybridization (Fig. 1 C). Western blot analysis of extracts of cultured fibroblasts from uPARAP/Endo180−/− 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 Mr 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 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−/− mice were born in a Mendelian ratio and were outwardly normal, viable, and able to reproduce. The observations documenting these conclusions are summarized in Fig. S1 and legend (available at http://www.jcb.org/cgi/content/full/jcb.200211091/DC1).

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−/− and uPARAP/Endo180+/+ 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−/− fibroblasts to internalize 125I-labeled collagen was determined and compared with the internalization of 125I-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−/− 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.

![Figure 2. uPARAP/Endo180 is required for collagen internalization.](http://www.jcb.org/cgi/content/full/jcb.200211091/DC1)
The cellular uptake of collagen I 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 Beetsen, 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 investi-
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 carry-
ing the disrupted uPARAP/Endo180 allele (Fig. 1 A), 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/DDBJ accession no. AAC52729) immunization of rab-
bits, affinity purification of antibodies and Western blotting of cell lysates
were performed as described previously (Schnack Nielsen et al., 2002) us-
ing the antibody at a final concentration of 2 μg/ml.

Ligand internalization assay
Primary skin fibroblasts were isolated from neonates as described previ-
ously (Holmbeck et al., 1999). 20 μg type I collagen, type IV collagen,
type VII collagen, and human heparin/transferin (all from Calbiochem-
Novacoh, and human plasma fibronectin (Sigma-Aldrich) were labeled with
125I as described previously (Behrendt et al., 1996). Cellular ligand in-
ternalization assays were performed as described previously (Hahn-Dan-
ton et al., 2001). In brief, samples of 104 cells were seeded in 24-well tis-
 nue culture plates and cultured in DME, 10% FCS until near confluence.
The cells were washed gently in DME at 37°C and were then cultured for
at least 1 h in binding buffer (DME, 20 mM Hepes, pH 7.4, 15 mM MgSA,
and 1X Nutridoma®SP) serum substitute (Roche). The medium was re-
moved 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 (G235–1; both from BD Biosciences) before the addition of la-
beled ligand. The cells were then washed twice with ice-cold PBS, and in-
cubated <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 mater-
ial) 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 (Kjøller and Hall, 2001), using the vector pcDNA3-uPARAP/
Endo180 (provided by Dr. Clare Isacke, Chester Beatty Laboratories, Lon-
don, UK; Sheikh et al., 2000). The transfection efficiency, estimated by de-
termination 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
Indiana University, Indiana, IN), human type I collagen, murine type II col-
lagen, human type IV collagen, or human type V collagen (all from Calbio-
chem-Novabiochem), 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 re-
cover from trypsinization. Cell viability was determined by Trypan blue
exclusion. 5 x 104 cells were added to each well and were allowed to at-
tach 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 prein-
cubated 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
coated collagen fibrils was verified by incubation with purified trypsin and MAB-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 (Cu et al., 1999). In brief,
cell movements were recorded using inverted microscopes (Cen-
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 Bonferroni multiple comparisons post-test were per-
formed 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 litter-
mate-matched uPARAP/Endo180+ litterate 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% mM MgCl2, 1 mg/ml
Na2N3, and 5 mg/ml BSA), and incubated for 30 min at 4°C with either
FITC-conjugated anti-β1 integrin antibody (HA2/5) or FITC-conjugated is-
type-matched control anti-TNP antibody (G235–1), using 5 μg of antibody
for 104 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 Na2N3. Flow cytometry analysis was then performed using a FAC-
sort® instrument (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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