Uptake of denatured collagen into hepatic stellate cells: evidence for the involvement of urokinase plasminogen activator receptor-associated protein/Endo180

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Tissue remodelling is dependent on the integration of signals that control turnover of ECM (extracellular matrix). Breakdown and endocytosis of collagen, a major component of the ECM, is central to this process. Whereas controlled secretion of matrix-degrading enzymes (such as matrix metalloproteinases) has long been known to mediate ECM breakdown, it is becoming clear that uPARAP/Endo180 (where uPARAP stands for urokinase plasminogen activator receptor-associated protein) serves as a receptor that mediates endocytosis of collagen by several types of cells. In the liver, the stellate cells play a major role in turnover of ECM including collagens. These cells synthesize various collagens and also produce matrix metalloproteinases. In the present study, we investigated the capacity of rat hepatic stellate cells to endocytose and degrade ^{125}I-labelled heat-denatured collagen I. It was found that the collagen is efficiently taken up and degraded by these cells. Degradation was inhibited by inhibitors of lysosomal proteases (leupeptin and E-64d) and the vacuolar proton pump (concanamycin A), indicating that it takes place in lysosomes. Furthermore, endocytosed FITC-labelled collagen was shown to reach late endocytic compartments in which it co-localized with LysoTracker (a marker of late endocytic compartments). Competition experiments showed that uPA and unlabelled collagen are capable of inhibiting binding and uptake of [^{125}I]collagen in a dose-dependent manner. Moreover, Western-blot analysis of cell lysate (using a polyclonal rabbit human-Endo180 antiserum) revealed a single band at 180 kDa. In addition, the antiserum was capable of reducing [^{125}I]collagen binding to the cell surface. Finally, using two primers designed from the human uPARAP/Endo180 mRNA sequence, the expression of uPARAP/Endo180 mRNA was detected by reverse transcriptase–PCR. These results together suggest that uPARAP/Endo180 mediates endocytosis of collagen in rat liver stellate cells.

Key words: collagen receptor, denatured collagen, lysosomal degradation, receptor-mediated endocytosis, urokinase plasminogen activator receptor-associated protein (uPARAP).

INTRODUCTION

Hepatic stellate cells are located within the perisinusoidal space between the basolateral surface of hepatocytes and the endothelial cells [1]. They are relatively scarce, $<10\%$ of the total resident liver cells are stellate cells [2]. A number of important functions have been attributed to stellate cells. They store vitamin A, received from the neighbouring hepatocytes [3,4], and, since they encircle the hepatic sinusoids and have contractile properties, they may regulate blood flow through the sinusoids [5–7]. They secrete cytokines and express a number of receptors that play a central role in cell–matrix and cell–cell interactions, both in normal and in injured liver [2,8]. The stellate cells exhibit two main phenotypes, the ‘quiescent state’ and the ‘activated state’. The quiescent cells have low proliferation rate and low fibrogenic activity, but after any injury to the liver the cells shift towards a myofibroblastic-like phenotype [2,5,8]. A similar activation process is observed when primary stellate cells are kept in culture on plastic support [9,10]: the cells elongate, lose their vitamin A droplets and develop myofilaments in their cytoplasm [5,8]. The activated cells gain functions such as proliferation and produce various growth factors and most of the ECM (extracellular matrix) components [9–12]. Activated stellate cells also produce enzymes that can degrade the normal pericellular ECM before synthesis of fibrous tissue. Such enzymes include MMPs (matrix metalloproteinases), a family of zinc-dependent enzymes [13,14]. Although activated stellate cells produce several factors needed for ECM degradation, it is conceivable that they in addition may remove components of the ECM by means of endocytosis. Very few studies have focused on this process in stellate cells. It was shown by using an *in vitro* system that retinol-binding protein, released from hepatocytes may be internalized in the stellate cells by receptor-mediated endocytosis [4]. A similar mechanism may operate when stellate cells take up retinol-binding protein from blood [15].

The aim of the present study was to determine whether stellate cells are able to endocytose collagen, a main component of the ECM. To this end we used cultured rat stellate cells, and heat-denatured collagen I, labelled with ^{125}I, was chosen as a representative ligand. Initial experiments demonstrated that the stellate cells internalize and degrade [^{125}I]collagen, and that the degradation is reduced by inhibitors of lysosomal function, indicating that the cells are able to endocytose [^{125}I]collagen. Moreover, the binding of [^{125}I]collagen was shown to be saturable, indicating that it is receptor-mediated. Further investigations indicated that the receptor in question could be the surface receptor uPARAP/Endo180 [where uPARAP stands for urokinase plasminogen activator receptor-associated protein]. uPARAP/Endo180 was

Abbreviations used: CTLD, C-type lectin-like domain; ECM, extracellular matrix; [^{125}I]TC, [^{125}I]tyramine cellobiose; MMP, matrix metalloproteinase; OVA, ovalbumin; RT, reverse transcriptase; TCA, trichloroacetic acid; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, uPA receptor; uPARAP, uPAR-associated protein; V-ATPase, vacuolar H^+-ATPase.

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originally identified as a constitutively recycling surface receptor [16] and has recently been shown to function as a collagen receptor [17–20]. A large pool (70–90 %) of the total uPARAP/Endo180 is maintained in endosomal compartments and the small plasma membrane pool (10–30 %) is associated with clathrin-coated pits [16,21]. uPARAP/Endo180 has also been called uPARAP because it can form a ternary complex with uPAR-bound pro-uPA. Collagen can block the formation of this trimolecular complex [22]. uPARAP/Endo180 is a member of the mannose receptor family, which also comprises the mannose receptor, the phospholipase A_2 receptor and the DEC-205/gp200-MR6 receptor [23]. These proteins are large in size (175–200 kDa) and contain several distinct domains including an NH_2-terminal cysteine-rich domain, a fibronectin-like type II domain, followed by either eight or ten tandemly arranged CTLDs (C-type lectin-like domains, named CTLD1–CTLD10), a single transmembrane domain and a short cytoplasmic domain containing one or two endocytic motifs to direct their internalization through clathrin-coated pits [23,24]. The fibronectin-like type II domains of all family members are considered to have collagen-binding capacity [23], but only the mannose receptor (through its CTLD4) and uPARAP/Endo180 (through its CTLD2) have been shown to bind carbohydrates [25,26]. uPARAP/Endo180 has been identified in many cell types including monocyte-like U937 cells, vascular smooth muscle cells [22], fibroblasts, vessel endothelial cells, macrophages [16,24,26], osteoblasts [27] and chondrocytes of young mice [28]. However, as far as we know, uPARAP/Endo180 has not been reported to be present in stellate cells.

The present study presents data indicating that uPARAP/Endo180 may be the main receptor responsible for endocytosis of denatured collagen in activated rat hepatic stellate cells.

EXPERIMENTAL

Materials

Type I calf skin collagen and type IV collagen were from Sigma and they were heat denatured by incubation at 60 °C for 30 min. High molecular mass urokinase was from Diagnostica & Analys Service AB (Göteborg, Sweden). tPA (tissue plasminogen activator) and E-64d were from Calbiochem (Oslo, Norway). Anti-rat CD49b (integrin $\alpha 2$ chain) and anti-rat CD29 (integrin $\beta 1$ chain) were from PharMingen International (Laborel AS, Oslo, Norway). The tetrapeptide Asp-Gly-Glu-Ala and the tripeptide Arg-Gly-Asp were from Bachem (Bubendorf, Switzerland). ^{125}I was from Amersham Biosciences. Polyclonal rabbit antibody to Endo180 was a gift from Dr C. M. Isacke (The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, U.K.). All other chemicals were purchased from Sigma unless otherwise mentioned.

Isolation and culture of stellate cells

Hepatic parenchymal and non-parenchymal cells were isolated from male Wistar rats, weighing 250–300 g, by the collagenase perfusion method [29,30]. After the separation of parenchymal cells by low-speed centrifugation at 50 ***g*** for 2 min, the supernatant containing stellate cells, other non-parenchymal cells and contaminating parenchymal cells were further centrifuged in a Percoll density gradient to isolate stellate cells [31]. After primary culture in plastic flasks using Dulbecco's modified Eagle's medium (BioWittaker, MedProbe, Oslo, Norway) containing 10 % (v/v) fetal bovine serum and 2 mM L-glutamine and antibiotics (BioWhittaker) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2, the stellate cells were used for experiments at 5–10 passages. These stellate cells were in 'activated state', as judged by the presence of desmin and loss of lipid droplets [9,10]. Cells were suspended by trypsinization when confluent and maintained on flasks as described above. Endocytosis experiments were performed with cells grown on 6- or 24-well plates. After the cells had reached approx. 60–80 % confluence, the wells were washed once with serum-free medium and then incubated in 1 ml of serum-free medium containing 2 mM L-glutamine, and 1 % BSA (to block non-specific-binding sites) at 37 °C for 30 min before experiments were started. Studies of ligand binding were performed as described above with the exception that the medium contained 20 mM Hepes and the experiments were started after an additional incubation at 4 °C for 30 min. Binding of radiolabelled ligands to wells without cells in the presence of 1 % BSA was negligible (< 0.05 %).

Radiolabelling of ligands

Denatured collagen (100 μg in 100 μl of PBS) was incubated with 10 μl of ^{125}I (40 MBq) in an Iodogen-coated tube (IODO-GEN; Bio-Rad Laboratories). After 45–60 min, the labelled protein was separated from free $Na^{125}I$ using a Sephadex G-25 column (Amersham Biosciences). The specific activity of [^{125}I]collagen was 8×10^5 c.p.m./μg. OVA (ovalbumin) or denatured collagen was labelled with [^{125}I]TC ([^{125}I]tyramine cellobiose) as described previously [32]. The specific activities of [^{125}I]TC-collagen and [^{125}I]TC-OVA were 3×10^5 and 5×10^5 c.p.m./μg respectively.

Endocytosis experiments

For studies of endocytic uptake of [^{125}I]collagen, the cells were incubated with the labelled ligand at 37 °C. In experiments using inhibitors, cells were incubated for 15 min with inhibitors before the addition of [^{125}I]collagen. At indicated time-points duplicate 0.1 ml aliquots were taken from the medium of each well for the determination of the amount of acid-soluble radioactivity released to the medium, and the cells were gently washed with ice-cold PBS and scraped in lysis buffer (0.1 % SDS, 0.1 M NaOH). The samples were precipitated with TCA (trichloracetic acid) and BSA at final concentrations of 10 and 0.5 % respectively, and centrifuged at 1000 ***g*** for 10 min at 4 °C. Acid-soluble and acid-precipitable radioactivities were counted in a gamma counter. Cell-associated ligand was calculated by subtracting radioactivity measured at time zero from cell-associated radioactivity measured at later time points. To determine the actual amount of degraded ligand released to the medium from the cells, acid-soluble radioactivity in wells without cells was subtracted from values in corresponding wells with cells. Total cellular uptake is expressed as the sum of cell-associated [^{125}I]collagen and acid soluble [^{125}I]-collagen released to the medium.

Confocal fluorescence microscopy

Serum-free Dulbecco's modified Eagle's medium (120 μl), containing 50 000 cells, was seeded on poly(D-lysine)-coated cover glasses in 24-well plates for 60 min at 37 °C. After washing away non-adherent cells, the cells were incubated with the uptake medium (serum-free medium containing 5 μg/ml heat-denatured type IV collagen labelled with FITC and 1 % BSA) at 37 °C. Uptake was allowed to proceed for 15 min in the presence and absence of LysoTracker Red (Molecular Probes; 0.5 μM), after which the cells were washed several times with PBS. The cells incubated in the presence of LysoTraker were then fixed in 3 % (w/v) paraformaldehyde, whereas cells incubated with FITC-collagen alone were first chased for 90 min in ligand-free medium

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and thereafter for 30 min in the presence of LysoTracker. After fixation, cover glasses were washed in PBS and then mounted on to Mowiol. Images were collected using an IX81 confocal microscope (OLYMPUS, FLUOVIEW, FV1000). FITC was excited using an argon laser at 488 nm, whereas LysoTraker was excited using a helium–neon laser at 543 nm.

Ligand-binding experiments

Binding experiments were performed with cells incubated at 4 °C with [^{125}I]collagen in the presence of increasing concentrations of unlabelled denatured collagen. For competition experiments, the cells were preincubated in the absence or presence of competitive molecules for 60 min at 4 °C, then [^{125}I]collagen was added, and after incubation for 2 h at 4 °C, the wells were washed gently with cold PBS to remove unbound ligand, and the cells were scraped off in lysis buffer before the determination of surface-bound [^{125}I]-collagen. Non-specific binding was calculated by subtracting radioactivity measured in the presence of a 50-fold excess of unlabelled denatured collagen.

Preparation of cell membrane proteins

Hepatic stellate cell membrane fractions containing the integral membrane proteins were prepared using a membrane extraction reagent kit (Mem-PER; Pierce) according to the manufacturer's instructions.

Lysate preparation, electrophoresis and immunoblot analysis

For SDS/PAGE experiments, cells were allowed to detach from the culture flasks by incubating with PBS at 37 °C with occasional gentle agitation. The cells were pelleted by centrifugation and then lysed in lysis buffer (0.3 M NaCl, 20 mM Hepes, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.1 % Triton X-100 and protease inhibitors) at 4 °C. The whole lysates were centrifuged at 10 000 ***g*** for 2 min and the clarified lysates were diluted 1:1 in sample buffer [Tris buffer, pH 6.8, 2 % SDS and 10 % (w/v) sucrose], heated for 5 min and then separated by SDS/PAGE under non-reducing conditions. After transfer on to PVDF membranes (Millipore), the membranes were blocked in 5 % (w/v) skimmed milk, incubated with the primary antibody (polyclonal rabbit anti-human-Endo180, diluted 1:5000) for 1 h, and thereafter washed three times in PBS containing 0.1 % Tween 20, and incubated with the secondary antibody (mouse anti-rabbit IgG conjugated to horseradish peroxidase) for 1 h. Finally, the membranes were washed three times and protein bands were detected using enhanced chemiluminescence ECL® reagent (Amersham Biosciences).

Analysis of gene expression by RT (reverse transcriptase)–PCR

Total RNA was isolated using TRIzol® (Invitrogen) from rat hepatic stellate cells, rat Kupffer cells, rat liver endothelial cells, rat parenchymal cells and J774 mouse macrophage cell-line according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript RT (Invitrogen). After cDNA synthesis, 20 μl of mixture was diluted to a final volume of 50 μl and from this cDNA mixture 1 μl was used as template in PCR. Primers were designed using the Primer 3 Output program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify Endo180 in rat liver stellate cells from the human Endo180 mRNA sequence. The primers used were: for Endo180: 5′-CCTGGGCATGTATGAGTGTG-3′ and 5′-TGTCAGTCAGCTGGTCCTTG-3′, and for β-actin: 5′-AGCCATGTACGTAGCCATCC-3′ and 5′-TCTCAGCTGTGGTGGTGAAG-3′. Routine PCR was performed using DyNAzyme (Finnzymes, Espoo, Finland) according to the manufacturer's instructions and performed at the following conditions: 94 °C for 5 min, 35 cycles each 30 s at 94 °C, 60 °C for 30 s and 72 °C for 30 s. The PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced (GATC-Biotech, Konstanz, Germany). PCR marker was provided by New England Biolabs (Beverly, MA, U.S.A.).

RESULTS

Uptake and degradation of [^{125}I]collagen by stellate cells

In initial experiments, we examined the ability of stellate cells to take up and degrade the [^{125}I]collagen. Cells were incubated with [^{125}I]collagen at 37 °C for different time periods and uptake and degradation of ligand were determined as described in the Experimental section. As shown in Figure 1(A), stellate cells take up [^{125}I]collagen efficiently. After 1 h at 37 °C in the presence of [^{125}I]collagen, the cells had removed approx. 5 % of the available ligand from the medium. To determine whether the ligand was transported to lysosomes for degradation, acid soluble and acid precipitable radioactivities were determined in cells and medium. The results show that degraded ligand accumulated both inside the cells and in the medium. Release of acid soluble radioactivity to the medium started after a lag phase lasting approx. 15 min. Acid precipitable radioactivity in the cells increased for approx. 30 min and then levelled off, indicating that an equilibrium between transport to and degradation in lysosomes had been reached. Similar results were obtained when denatured ^{125}I-labelled collagen IV was used (results not shown). That degradation of collagen takes place intracellularly was verified by the finding that degradation products formed from [^{125}I]TC-collagen were not released to the medium during the incubation time (120 min) (results not shown). Labelled degradation products formed from this probe are trapped in the degradative compartment [33].

We also analysed the transport of fluorescently labelled denatured collagen to late endocytic compartments in pulse–chase experiments. Fluorescence microscopy using FITC-labelled collagen (Figure 2) showed lysosomal/late endosomal staining, as indicated by co-localization (yellow) due to overlap of the internalized ligand (green) and LysoTracker (red), a marker of acidic endocytic compartments.

Binding and internalization of [^{125}I]collagen to hepatic stellate cells is saturable and pH-dependent, but not calcium-dependent

To determine whether endocytosis of [^{125}I]collagen is receptor-mediated, cells were incubated with [^{125}I]collagen in the presence of increasing concentrations of unlabelled collagen. The results obtained (Figure 1B) show that both binding (at 4 °C) and uptake (at 37 °C) of [^{125}I]collagen were reduced by adding unlabelled collagen. At 6 μg/ml of unlabelled collagen, binding and uptake of [^{125}I]collagen were decreased by 70 and 90 % respectively. Taken together, these experiments indicate that the uptake of collagen represents a receptor-mediated process and not merely fluid-phase endocytosis. Binding of [^{125}I]collagen was pH-dependent, and was reduced by approx. 50 % when pH was reduced to pH 5.5 (results not shown). Binding was, however, retained in the presence of the calcium chelator EGTA (2.2 mM).

Degradation of [^{125}I]collagen is inhibited by protease and proton pump inhibitors

Leupeptin, an inhibitor of cysteine proteases, inhibited the degradation of [^{125}I]collagen partially (Figure 3, upper panel). After

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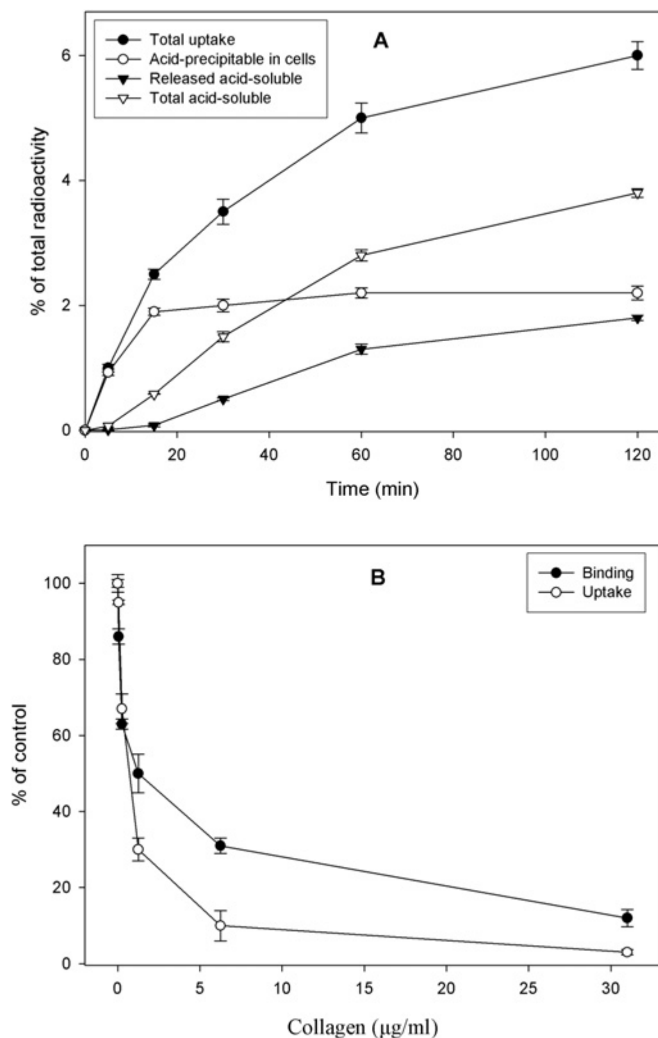

Figure 1 Uptake and binding of [^{125}I]collagen to hepatic stellate cells

(**A**) Time course of receptor-mediated uptake and degradation of collagen: cells (∼10^6 cells/well) were incubated with [^{125}I]collagen (0.2 μg/ml) at 37 °C for the indicated times, washed and then lysed. Cell lysates and medium were treated with TCA to determine acid-soluble and acid-precipitable radioactivities as described in the Experimental section. Values are expressed as percentage of total initial acid precipitable radioactivity in the medium and are means ± S.D. results for triplicate samples. Equivalent results were obtained in at least six separate experiments. (**B**) Concentration-dependent binding and uptake of collagen in stellate cells: cells were pre-incubated at 4 °C for 30 min with the indicated concentrations of unlabelled collagen. After preincubation, [^{125}I]collagen (20 ng/ml) was added and the cells were incubated for 120 min at 4 °C or 30 min at 37 °C. The cells were then washed three times and the cell-associated radioactivity was determined. Values are the means ± S.D. results for triplicate samples. Equivalent results were obtained in at least three separate experiments.

90 min of incubation at 37 °C, formation of acid soluble radioactivity was reduced by approx. 50 % in the presence of leupeptin (2 mM). Release of acid-soluble degradation products from the cells was, however, reduced by nearly 80 % by the inhibitor. These results suggest that the processing of internalized [^{125}I]collagen first produces acid-soluble peptides, which are then further degraded to amino acids (released from the cells). Leupeptin primarily inhibits degradation of the smaller peptides. Preventing collagen degradation caused, as expected, the accumulation of both acid-precipitable and acid-soluble radioactivity in the cells. We also assessed the effect of E-64d, a selective and irreversible inhibitor of lysosomal cysteine proteases, on the degradation of internalized [^{125}I]collagen. As indicated in Figure 3 (upper panel), treatment of cells with E-64d gave results similar to those seen with leupeptin treatment, although at a much lower concentration (50 μM) of the inhibitor. Lysosomal degradation of endocytosed ligands depends on a functional vacuolar H^+-ATPase (V-ATPase) in the endosomal/lysosomal compartments [34]. To determine whether the inactivation of V-ATPase affects collagen degradation, the cells were incubated in the presence of concanamycin A, a selective inhibitor of V-ATPase [35]. The results obtained (Figure 3, lower panel) show that concanamycin A inhibited both uptake and degradation of [^{125}I]collagen. In cells incubated for 90 min at 37 °C, the degradation of labelled collagen was reduced approx. 85 % in the presence of 0.1 μM concanamycin A. Uptake was reduced approx. 60 % at this drug concentration.

Effects of possible competitive ligands and anti-integrin antibodies on [^{125}I]collagen binding

The rapid removal of collagen from the culture medium suggested the existence of a specific receptor(s) in hepatic stellate cells. Several integrins have been reported to act as binding receptors for denatured collagen, including $\alpha2\beta1$ [36,37] and $\alpha v\beta3$ [38,39]. We therefore used anti-integrin antibodies and ligands of these receptors in competition experiments to see whether they were capable of competing with [^{125}I]collagen for binding. At high concentrations (> 20 μg/ml), the anti-integrin $\alpha2$ chain inhibited [^{125}I]collagen binding by approx. 10 %, whereas anti-integrin $\beta1$ chain did not significantly inhibit the binding of [^{125}I]collagen. No additional inhibition was observed using both antibodies together. Peptides containing the RGD (Arg-Gly-Asp) sequence have been shown to inhibit specifically the binding of denatured type I collagen to the $\alpha v\beta3$ integrin, possibly because this sequence becomes exposed on collagen denaturation [38], whereas the sequence DGEA (Asp-Gly-Glu-Ala) binds to $\alpha2\beta1$ integrin [40]. [^{125}I]Collagen binding was therefore examined in the presence of the tripeptide Arg-Gly-Asp and the tetrapeptide Asp-Gly-Glu-Ala. Neither of these peptides (> 0.5 mg/ml) significantly inhibited the binding of [^{125}I]collagen (results not shown). Scavenger receptors have also been shown to serve as receptors for denatured collagen [41]. No inhibitory effects on the binding and uptake of [^{125}I]collagen were, however, observed when cells were incubated with a 50 M excess of formaldehyde-treated BSA, a ligand of scavenger receptors [42] (results not shown).

uPA inhibits binding and uptake of [^{125}I]collagen

Collagen has been shown to block the association of Endo180 with uPAR-bound pro-uPA [22]. Conceivably, uPA might also bind to the collagen-binding receptor in the stellate cells if this receptor is related to the uPARAP/Endo180 receptor. It was found that uPA inhibits both binding and uptake of [^{125}I]collagen to the stellate cells in a concentration-dependent manner (Figure 4). At 3 mg/ml, uPA reduced both binding and uptake of labelled collagen by approx. 80 %. It may, however, be argued that the reduced binding of collagen in the presence of uPA is due to the activation of collagen-degrading enzymes (such as MMP-13) secreted by the cells into the medium during the 3.5 h incubation (30 min at 37 °C and 3 h at 4 °C). To rule out this possibility, similar experiments were performed in the presence of tPA, which binds receptors other than uPAR [43]. However, incubation with 2.2 mg/ml tPA did not inhibit significantly the binding of collagen to the cells (binding as percentage of total: 1.04 ± 0.19 and 0.96 ± 0.09; $n = 3$, for control and tPA respectively), indicating that the reduced binding of collagen is due to binding of uPA to its receptor (or

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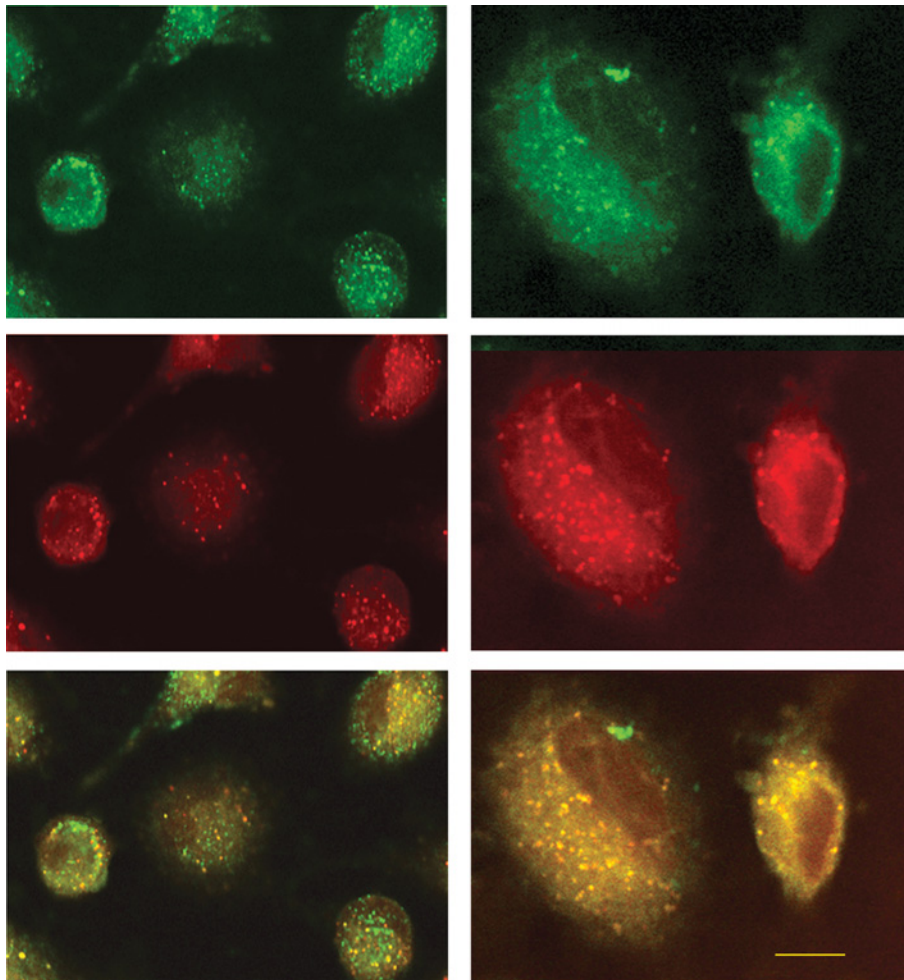

Figure 2 Fluorescence microscopy showing co-staining of the internalized FITC-collagen and a marker of late endocytic compartments

Cells were pulse-labelled at 37 °C for 15 min with FITC-collagen (green) in the presence or absence of LysoTracker (red), then washed and either fixed (images on the left) or chased for 90 min, after which the incubation continued for an additional 30 min in the presence of LysoTracker (images on the right). Co-localization of FITC-collagen with LysoTracker (yellow) is shown in the merged images (lowest panels). Results represent two or three experiments.

to uPARAP/Endo180, see below) and not to an indirect effect of uPA on the activation status of collagenases in the medium.

uPARAP/Endo180 is expressed in rat hepatic stellate cells

Result from experiments with uPA suggested that uPARAP/Endo180 is probably involved in the uptake of collagen. Therefore we performed Western-blot analysis, using a polyclonal antibody against purified human uPARAP/Endo180 [16]. U937 cells, the cell line originally used to define uPARAP activity [22], were used as positive controls, and HepG2 cells were used as negative controls since they do not express uPARAP/Endo180 [24]. As shown in Figure 5, no band was detected in the preparation from HepG2 cells, whereas a specific band at 180 kDa was revealed in preparations from both hepatic stellate cells and U937 cells. We also tested the ability of the Endo180 antiserum to interfere with collagen binding to the cells and found that the binding of [^{125}I]collagen to the stellate cells was significantly reduced by 50 % when 20 μl/ml of the antiserum was used (results not shown). Analysis by RT–PCR showed that uPARAP/Endo180 is abundantly expressed in stellate cells, but is not detectable in Kupffer cells, liver endothelial cells, hepatocytes and J774 cells (Figure 6). The PCR product gave the expected length of 422 bp when compared with the human uPARAP/Endo180 sequence from which the primers were designed (AF134838). The PCR product was sequenced and shown to have a homology of 89 % with the human uPARAP/Endo180 (AF107292, NM_006039 and AF134838) and 94 % homology was seen with the mouse mannose receptor uPARAP/Endo180 receptor (NM_008626) as well. The β-actin PCR products (227 bp) showed the same mRNA content in all cell types tested.

Uptake of OVA

CTLD2 in Endo180 has been shown to contain a Ca^{2+}-dependent carbohydrate-binding determinant, which specifically binds to *N*-acetylglucosamine, mannose and fucose [26]. To determine whether hepatic stellate cells express such binding activity, we measured the uptake of OVA (which contains sugars terminating in *N*-acetylglucosamine and mannose [44]). It was found that the stellate cells are also capable of taking up OVA. As shown in Figure 7, uptake and degradation of [^{125}I]TC-OVA increases as a function of time. Moreover, the uptake (measured after 30 min incubation at 37 °C) was reduced by 90 ± 4 and 87 ± 5 % by the

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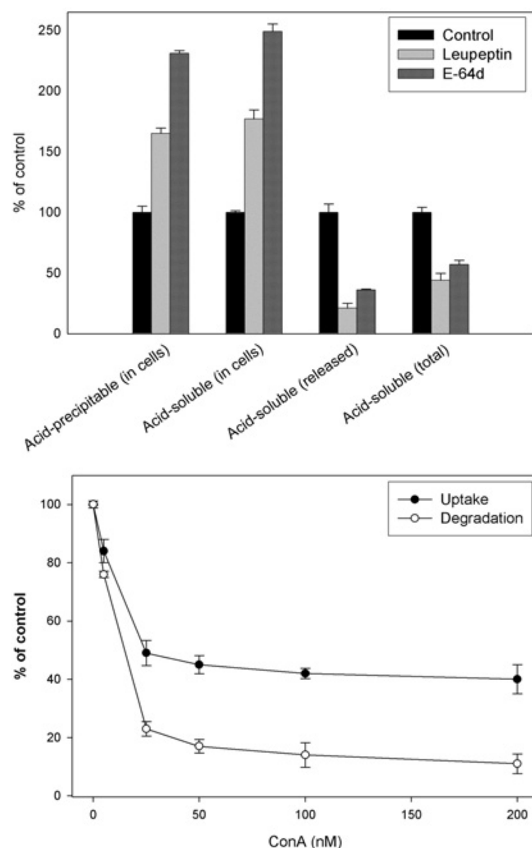

Figure 3 Effect of leupeptin, E-64d and concanamycin on uptake and degradation of [^{125}I]collagen

Cells were preincubated with either leupeptin (2 mM), E-64d (2 μM) (upper panel) or increasing concentrations of concanamycin (lower panel) for 15 min at 37 °C. After preincubation, radiolabelled collagen (40 ng/ml) was added, followed by 90 min incubation at 37 °C and three washes. Cell lysates and medium were treated with TCA to determine acid-soluble and acid-precipitable radioactivities as described in the Experimental section. Values are expressed as percentage of controls and are the means ± S.D. results for triplicate samples. Equivalent results were obtained in at least three separate experiments.

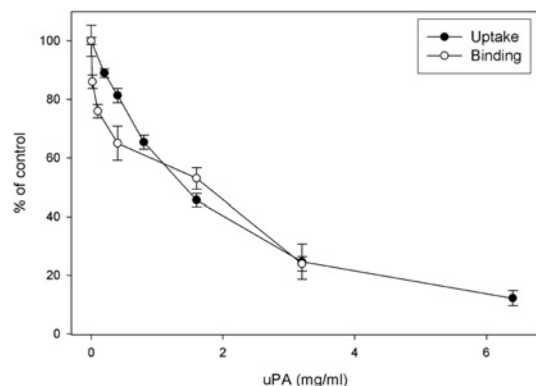

Figure 4 Dose-dependent inhibition of binding and uptake of [^{125}I]collagen to stellate cells by uPA

Cells were preincubated with increasing concentrations of uPA for 30 min at 4 °C. After preincubation, radiolabelled collagen (20 ng/ml) was added, followed by 120 min incubation at 4 °C and three washes. For uptake, uPA was added just before the addition of [^{125}I]collagen and cells were incubated at 37 °C for 30 min. Results are means ± S.D. of a representative experiment performed in triplicate.

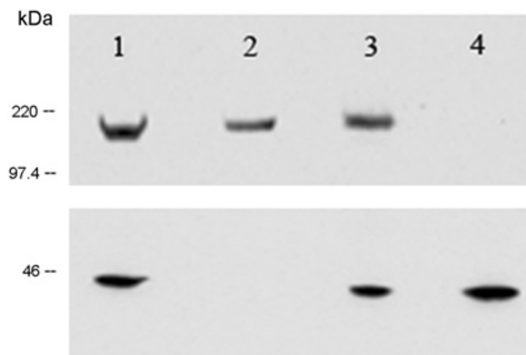

Figure 5 Endo180 is expressed in rat hepatic stellate cells

The whole lysates (40 μg) of hepatic stellate cells (lane 1), U937 cells (lane 3) and HepG2 cells (lane 4) were analysed by SDS/PAGE on a 6 % gel and then transferred on to membrane. The blot was incubated first with a polyclonal antiserum directed against Endo180 and subsequently with mouse anti-rabbit IgG conjugated to horseradish peroxidase followed by detection with ECL®. Lane 2 corresponds to the cell membrane fraction (20 μg) prepared from stellate cells. The membrane was then stripped, blocked and incubated with anti-β-actin. Relative molecular mass standards are shown on the left (masses in kDa). The results shown were reproduced in at least three independent experiments.

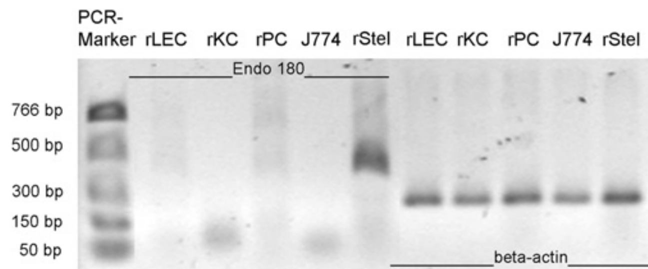

Figure 6 Expression analysis of uPARAP/Endo180 mRNA by RT–PCR

Four different types of rat liver cells [liver endothelial cells (rLEC), Kupffer cells (rKC), parenchymal cells (rPC) and stellate cells (rStel)] and J774 mouse macrophages were tested for mRNA expression of Endo180 (lanes 2–6 respectively) with β-actin mRNA as an internal control (lanes 7–11). Three experiments gave equivalent results. Sizes are indicated in bp.

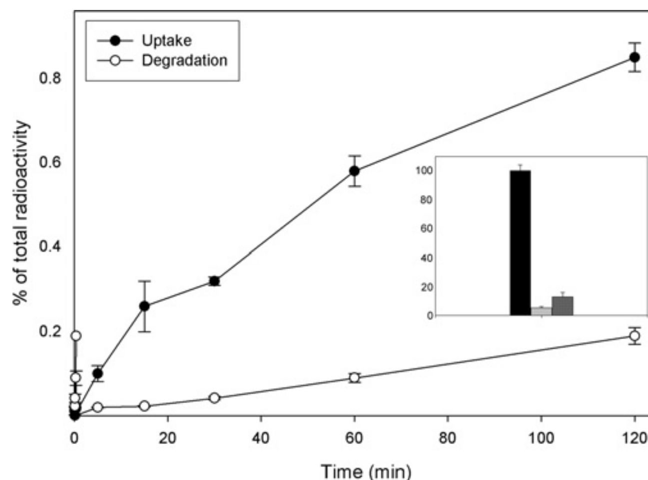

Figure 7 Uptake and degradation of [^{125}I]TC-OVA by hepatic stellate cells

Cells (~10^6 cells/well) were incubated at 37 °C with 2 nM [^{125}I]TC-OVA in the presence and absence of a 50-fold excess of unlabelled OVA or 2.2 mM EGTA. At timed intervals the cells were washed and cell-associated radioactivity (acid-soluble and acid-precipitable) was measured as described in the Experimental section. Inset: the effect of unlabelled OVA and EGTA on the uptake.

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addition of 50-fold excess of unlabelled OVA and 2.2 mM EGTA respectively (Figure 7, inset).

DISCUSSION

The present results demonstrate that the uptake of heat-denatured ^{125}I-labelled collagen in rat hepatic stellate cells is mediated by a saturable binding site. To identify the receptor(s) involved, we measured the uptake of [^{125}I]collagen in the presence and absence of antibodies and ligands that bind to potential receptor candidates. The results obtained seemed to exclude integrins ($\alpha 2\beta 1$ and $\alpha v\beta 3$) and scavenger receptors. The discoidin domain receptors (DDR1 and DDR2) are, probably, not involved in binding, as these receptors require the native, triple-helical configuration of the collagen [45,46]. On the other hand, several observations pointed to the uPARAP/Endo180 as the receptor involved in binding collagen. Behrendt et al. [22] found that collagen prevents the formation of a complex between pro-uPA-uPAR and uPARAP/Endo180. We found that the reciprocal interaction, the interaction of uPA-uPAR with uPARAP/Endo180, prevents uPARAP/Endo180 from binding to collagen. Alternatively or in addition, uPA can directly bind to Endo180. This result suggested that [^{125}I]collagen binding in stellate cells could be mediated by the uPARAP/Endo180. That the receptor in question really is uPARAP/Endo180 was verified using Western-blot analysis and RT–PCR. Moreover, collagen binding was significantly reduced by Endo180 antiserum, strongly supporting the involvement of uPARAP/Endo180 in mediating the uptake of collagen. Taken together, these observations are compatible with the newly defined role of uPARAP/Endo180 as an endocytic collagen receptor [17–20]. The stellate cells also bind [^{125}I]TC-OVA, compatible with the observation that uPARAP/Endo180, which belongs to the mannose-receptor family, can also function as a lectin, binding ligands with terminal mannose, fucose and *N*-acetylglucosamine [26]. Moreover, the binding of the OVA is calcium-dependent, whereas the binding of collagen is not. This is in agreement with earlier studies of the uPARAP/Endo180 receptor [18].

Inhibition of [^{125}I]collagen degradation by leupeptin and E-64d, two inhibitors of lysosomal thiol proteinases and concanamycin A, an inhibitor of the vacuolar proton pump [35,47], shows that the ligand is effectively transported to late endocytic compartments for degradation. These observations are in agreement with recent reports demonstrating that uPARAP/Endo180 mediates endocytosis and lysosomal degradation of collagens in fibroblasts [20] and in a breast cancer cell line (MCF-7) [18]. To the best of our knowledge, the present study is the first to demonstrate receptor-mediated endocytosis of collagen in hepatic stellate cells.

The affinity of the uPARAP/Endo180 for denatured collagen is reduced at pH $\leqslant 6.5$ and the finding that the proton pump inhibitor also reduces cellular uptake of [^{125}I]collagen is probably a result of increases in pH in early or late endosomes. This will prevent dissociation of [^{125}I]collagen from the collagen receptor in endosomes, and the ligand will recycle to the plasma membrane bound to the receptor. uPARAP/Endo180 has been regarded as a constitutively recycling surface receptor.

A collagen receptor in activated stellate cells may play a crucial role in the turnover of ECM in liver. The hepatic stellate cells are the major producers of ECM both in normal liver and in the injured liver [48,49]. They synthesize a large variety of ECM components [2,5,8] and also participate very actively in their degradation. To this end different calcium- and zinc-dependent enzymes are produced, each being specific for a group of matrix components. MMPs comprise collagenases, gelatinases, strombelysins and metalloelastases. The stellate cells are the main source of MMP synthesis during liver fibrosis [2,9,50]. The main function of the uPARAP/Endo180 may be to mediate endocytosis of partially degraded collagens (and glycoproteins) produced in the ECM by MMPs. MMP-mediated cleavage of fibrillar collagen results in altered structure accompanied by rapid denaturation [51]. These denatured fragments may be treated by uPARAP/Endo180 *in vivo* as heat-denatured collagen is *in vitro*. The uPARAP/Endo180 in stellate cells would thereby co-operate with the secreted MMPs in the remodelling of the matrix, especially during the recovery phase after liver injury.

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