The shed ectodomain of type XIII collagen affects cell behaviour in a matrix-dependent manner

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Transmembrane type XIII collagen resides in adhesive structures of cells and tissues, and has therefore been implicated in cell adhesion and in adhesion-dependent cell functions. This collagen also exists as a soluble protein in the pericellular matrix, as the ectodomain is released from the plasma membrane by proteolytic cleavage. Analysis with various protease inhibitors led to confirmation of the furin family of proprotein convertases as the protease group responsible for the shedding of the ectodomain, cleaving at a site conforming to the consensus sequence for the proprotein convertases at the stem of the ectodomain. Both the *trans*-Golgi network and the plasma membrane were used as cleavage locations. Mammalian cells employed various intracellular mechanisms to modulate shedding of the ectodomain, all resulting

in a similar cleavage event. Cell detachment from the underlying substratum was also found to augment the excision. The released ectodomain rendered the pericellular surroundings less supportive of cell adhesion, migration and proliferation, as seen specifically on a vitronectin substratum. Type XIII collagen ectodomain shedding thus resulted in the formation of a soluble, biologically active molecule, which eventually modulated cell behaviour in a reciprocal and substratum-specific manner. The dual existence of membrane-bound and soluble variants widens our biological understanding of type XIII collagen.

Key words: collagen, ectodomain, proprotein convertase, shedding, transmembrane, vitronectin.

INTRODUCTION

The extracellular domains (ectodomains) of an increasing number of integral membrane proteins are liberated from the plasma membrane into the surrounding pericellular milieu by specific proteolytic cleavage. The diversity and scope of such proteins is remarkable, encompassing practically all functional classes of membrane proteins. For the cells, shedding of the ectodomain may represent a means of ridding themselves of a spent protein. Alternatively, controlled release is seen as an important regulatory mechanism for optimizing the protein content of the plasma membrane and/or for generating soluble, biologically active molecules capable of modifying the neighbouring microenvironment. The dual existence of a protein in both membrane-bound and soluble isoforms allows the cells to multiply the functional roles of proteins in a cost-efficient manner without any need of creating a separate biosynthetic pathway for the soluble forms [1–3].

Ectodomains are released from the plasma membrane by cleavage at specific amino acid sequences, mainly by members of the ADAM (a disintegrin and metalloproteinase) and MMP (matrix metalloproteinase) families, with the serine proteinases also implicated [2–6]. Once soluble, ectodomains may possess greatly altered properties relative to their membrane-bound ancestors, resulting from the activation of an earlier dormant molecule, for instance, or the exposure of cryptic functional neo-epitopes with different ECM (extracellular matrix) ligand-binding activities [2,7,8].

Transmembrane type XIII, XVII, XXIII and XXV collagens are all known to shed ectodomains [9–13]. The type XVII collagen

ectodomain was originally presumed to be cleaved by furin [10,14], but the ADAMs TACE (tumour necrosis factor- α convertase), ADAM-9 and ADAM-10 have been shown more recently to be actively involved [15]. The ectodomains of type XXIII and CLAC-P (precursor of the collagen-like Alzheimer amyloid plaque component)/type XXV collagens are released by furin convertase activity at sites conforming to the furin consensus sequence [12,13]. Type XIII collagen is an integral membrane protein residing in focal adhesions, on account of which it is implicated in modulating cell adhesion [16,17]. It is also known to be present as a secreted soluble protein in insect cell cultures, presumably through furin-driven proteolytic cleavage of the ectodomain [11].

Furin belongs to the mammalian subtilisin-like PCs (proprotein convertases) of calcium-dependent serine endoproteases [18]. This protease family comprises seven members, namely PC1/PC3, PC2, furin, PC4, PACE4 (paired basic amino-acid-converting enzyme 4), PC5/PC6 and PC7/SPC7 (subtilisin-like PC7)/LPC (lymphoma PC), of which furin is the most studied so far. Some of these PCs show a restricted tissue or developmental expression pattern, whereas others, such as furin, PACE4 and PC7, are ubiquitously expressed in all mammalian cells. Subcellularly, these PCs are located predominantly in the TGN (*trans*-Golgi network), while also recycling to the plasma membrane via the endosomal pathway [19,20]. Considered to localize within the constitutive secretory pathway, these PCs recognize and cleave the target proteins on the C-terminal side of the consensus cleavage motif of Arg/Lys-Xaa_n-Arg/Lys, where *n* = 0, 2, 4 or 6 amino acid residues, and are functionally redundant in the processing of proteins [19–21]. Since soluble type XIII collagen

Abbreviations used: ADAM, a disintegrin and metalloproteinase; BFA, brefeldin A; CM, conditioned medium; decRVKRCmk, decanoyl-Arg-Val-Lys-Arg-chloromethane; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EHS, Englebreth-Holm-Swarm; FHC cells, foetal human colon cells; MMP, matrix metalloproteinase; NC3, non-collagenous domain #3 of type XIII collagen; PACE4, paired basic amino-acid-converting enzyme 4; PC, proprotein convertase; PKC, protein kinase C; TACE, tumour necrosis factor- α convertase; TAPI-1, tumour necrosis factor- α protease inhibitor; TGN, *trans*-Golgi network.

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ectodomain has been found recently to associate with a number of ECM proteins *in vitro* [22], our aims in the present study were to characterize the shedding process in mammalian cells more closely, and to address the functional aspects and biological significance of the released ectodomain *in vivo*.

MATERIALS AND METHODS

Cell culture, expression plasmid and transfection

Human primary dermal fibroblasts and HT-1080 cells were grown in DMEM (Dulbecco's modified Eagle's medium (Biochrom KG), human SW-480 cells (CCL-228; A.T.C.C.) in Leibovitz's L-15 medium (Gibco) and human LoVo cells (CCL-229, A.T.C.C.) in Ham's F12K medium (BioWhittaker Europe). Human intestinal FHC (foetal human colon) cells (CRL-1831; A.T.C.C.) were grown in a 1:1 mixture of Ham's F12 medium and DMEM containing 25 mM Hepes, 10 ng/ml cholera toxin (Sigma), 5 µg/ml insulin (Sigma), 5 µg/ml transferrin (Sigma) and 100 ng/ml cortisol (Sigma). All media were supplemented with 10 % foetal bovine serum (EuroClone). SW-480 cells were incubated without CO₂. The plasmid COLXIII for the transfections was created in a mammalian expression pcDNA3.1(–)/Myc-HisB vector (Invitrogen) by ligating *NotI/EcoRI*-digested full-length type XIII collagen cDNA lacking the stop codon, which was eliminated by site-directed mutagenesis (GeneEditor™ *in vitro* Site-Directed Mutagenesis System; Promega) to include an in-frame Myc tag from the expression vector. Cells were counted with a Coulter™ cell counter (Coulter) and transfected with FuGENE6 transfection reagent (Boehringer) for 48 h.

Coating

Fibronectin, vitronectin, EHS (Englebreth–Holm–Swarm) laminin, poly-D-lysine and collagen types I and IV were from Becton Dickinson Collaborative Biochemical Products. The recombinant human type XIII collagen ectodomain was purified as described in [22]. BSA was from Serva/Invitrogen Life Technologies. The 96-well plates (MaxiSorp™; Nunc Nalge International) and Boyden chambers (Transwell®; Costar) were coated with 5 µg of protein/cm².

Antibodies

The production of anti-(type XIII collagen NC3 domain) antibody, where NC3 is non-collagenous domain #3 of type XIII collagen, is described in [16]. The antibodies used were as follows: anti-c-Myc oncoprotein (clone 9E10.3) from NeoMarkers, anti-actin (I-19) and anti-α5 integrin (H-104) from Santa Cruz Biotechnologies, and horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse and anti-goat antibodies from BioRad.

Chemicals

PMA, BFA (brefeldin A), A23187, monensin and 1,10-phenanthroline were purchased from Sigma, the protease inhibitor kit from Boehringer Mannheim, TAPI-1 (tumour necrosis factor-α protease inhibitor) from Calbiochem, GM6001 from Chemicon, leupeptin and aprotinin from Roche, decanoyl-Arg-Val-Lys-Arg-chloromethane (decRVKRCmk) from Bachem, methanol from Riedel-de Haën (Seelze, Germany) and EDTA from Fluka. Pervanadate was prepared before use by mixing equal volumes of 2 M sodium orthovanadate (Sigma-Aldrich) and 1 M H₂O₂ (Merck) [23].

Whole-cell shedding assay

Before the shedding assay, cells were plated to subconfluence and the serum-containing medium was replaced by a serum-free medium and supplemented with the desired agent for a given period of time. The shed ectodomain was precipitated from the medium at –20 °C by adding an equal volume of ice-cold methanol, collected by centrifugation at 1800 g for 30 min and suspended in a lysis buffer containing 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 % (v/v) Triton X-100 and 20 mM Tris, pH 7.6. The cells were detached by scraping, suspended in the lysis buffer and homogenized manually. The protein concentrations were determined using a BCA (bicinchoninic acid) Protein Assay Kit (Pierce).

Cell-surface biotinylation and streptavidin pull-down

Cell-surface biotinylation was performed before the shedding assay with a FluoReporter Cell-Surface Biotinylation Kit (Molecular Probes) for equal cell numbers in the various treatments. The HT-1080 cell membranes were fractionated as described in [16]. The streptavidin pull-down assay with avidin-conjugated Protein A–Sephrose (Amersham Biosciences) was modified from that described in [4,24]. Protein concentrations were determined as described above to ensure equal loading. The samples were boiled in a buffer containing 1 % (v/v) Triton X-100 and 0.1 % (w/v) SDS before Western blotting analysis.

Western blotting

Equal amounts of protein were fractionated by SDS/PAGE (8 or 10 % gels). The antibodies used are listed above. ECL® (enhanced chemiluminescence; Amersham Bioscience) or Lumilight (Hoffman-La Roche) Western Blotting Detection Kits were used for detection. A BioRad GS-710 densitometer with QuantityOne-4.2.2. software was used for quantification.

Cell adhesion assay

Fibroblasts (3×10^4) were plated in serum-free medium on 96-well cell-culture plates coated with vitronectin, fibronectin, laminin or collagens type I, IV or XIII ectodomain, followed by incubation for 3 h to permit possible weak cell adhesion. Cell adhesion was determined with a CyQuant Cell Proliferation Kit (Molecular Probes) and quantified using a 1420 Victor™ multi-label plate reader (PerkinElmer Life Sciences/Wallac).

Cell migration assays

Two migration assays were employed: for haptotactic cell migration [25,26] to determine how the ectodomain supports cell migration, Boyden chamber inserts with 8 µm diameter membrane pores were coated with fibronectin, laminin, collagen type I and type XIII collagen ectodomain. Fibroblasts (5×10^4) were plated in serum-free culture medium, and permitted to transmigrate for 3 h, after which those on the upper side of the membrane were removed with a cotton swab. The membranes were fixed with 1 % (w/v) paraformaldehyde, permeabilized in ethanol, stained with haematoxylin and mounted on microscope slides. In the scratch wound assay, denuded areas were created by scraping the monolayer cell cultures. After washing, these cells were supplemented with 0.5 µM purified ectodomain in a serum-free medium or serum-free CM (conditioned medium) from HT-1080 cells, and allowed to migrate over the denuded area for 4

Figure 1 Characterization of the size of the shed ectodomain

The media of human primary fibroblasts, FHC intestinal epithelial cells and HT-1080 fibrosarcoma cells containing the shed ectodomain were precipitated and analysed by SDS/PAGE (8% gels), followed by detection with anti-(type XIII collagen NC3 domain) antibody. The ectodomain size comparison was carried out on the respective cell lysates containing the uncleaved type XIII collagen. Migration of the molecular-mass markers (kDa) is shown on the left. L, lysate; M, medium.

or 24 h, after which they were photographed with conventional optics.

Cell proliferation assay

Fibroblasts (5×10^3) in serum-free medium (Fibroblast Growth Medium; PromoCell, Heidelberg, Germany) were plated on vitronectin- or fibronectin-coated 96-well plates, and allowed to grow for 4, 24, 48 or 72 h, with daily changes of the culture medium, supplemented with $0.5 \mu\text{M}$ purified ectodomain or BSA. Cell proliferation was determined with a CyQuant Cell Proliferation Kit. Cell phenotypes were monitored by photographing with a phase-contrast microscope at 4 and 24 h.

RESULTS

The shed ectodomain is of similar size irrespective of the cell line

Human primary skin fibroblasts, FHC intestinal epithelial cells and HT-1080 fibrosarcoma cells were used to characterize the size of the shed ectodomain in mammalian cells. The cleaved ectodomain, precipitated from serum-free medium and analysed by Western blotting with anti-(NC3 domain) antibody, was found to migrate as a slightly smaller fragment than the intact type XIII collagen of the cell lysates. The size difference between the two was estimated to be approx. 10 kDa, irrespective of the cell line (Figure 1).

Protease inhibitors suggest the involvement of PCs

A series of protease inhibitors were tested for blocking of ectodomain shedding in HT-1080 cells. Serum-free shedding assays were supplemented with decRVKRCmk ($80 \mu\text{M}$), antipain ($50 \mu\text{g/ml}$), bestatin ($30 \mu\text{g/ml}$), chymostatin ($60 \mu\text{g/ml}$), E-64 ($10 \mu\text{g/ml}$), leupeptin ($10 \mu\text{M}$), pepstatin ($1 \mu\text{M}$), phosphoramidon ($300 \mu\text{g/ml}$), EDTA (1 mM), aprotinin ($0.3 \mu\text{M}$) or EGTA (1 mM). Western blotting analysis of the precipitated media with anti-(NC3 domain) antibody showed that effective blocking of shedding in the HT-1080 cells was seen only with decRVKRCmk (Figure 2).

The potential involvement of MMPs or ADAMs in the ectodomain shedding was excluded by the lack of inhibitory effect on the shedding by the broad-spectrum hydroxamic-acid-derived inhibitors, 1,10-phenanthroline, GM6001 or TACE inhibitor TAPI-1

Figure 2 Effect of protease inhibitors on shedding

HT-1080 cell cultures were supplemented with protease inhibitors, including $80 \mu\text{M}$ decRVKRCmk, $50 \mu\text{g/ml}$ antipain, $30 \mu\text{g/ml}$ bestatin, $60 \mu\text{g/ml}$ chymostatin, $10 \mu\text{g/ml}$ E-64, $10 \mu\text{M}$ leupeptin, $1 \mu\text{M}$ pepstatin, $300 \mu\text{g/ml}$ phosphoramidon, 1 mM EDTA, $0.3 \mu\text{M}$ aprotinin and 1 mM EGTA. The untreated control is shown on the far left. The precipitated media were analysed by SDS/PAGE (8% gels), followed by detection with anti-(NC3 domain) antibody. The histogram represents densitometric measurements of the Western blot bands of the precipitated media relative to the untreated control, set at 100%. Intact type XIII collagen and actin in the cell lysates were detected by anti-(type XIII collagen NC3 domain) and anti-actin antibodies. Actin was used as a loading control.

tested in the whole-cell shedding assays (results not shown). Shedding specifically at the cell membrane by MMPs, ADAMs or active plasmin was ruled out, as the cell-surface-biotinylated HT-1080 cells released ectodomain in spite of 1,10-phenanthroline, GM6001 or plasmin inhibitors aprotinin and leupeptin (results not shown).

LoVo cells deficient in furin still shed the ectodomain

Furin-deficient LoVo cells expressing only PC7 and PACE4 [27,28] were used to evaluate the involvement of the PCs in ectodomain shedding. The low endogenous expression of type XIII collagen in LoVo cells necessitated their transfection with an expression plasmid encoding a Myc-tagged recombinant type XIII collagen before the shedding assay. The transfected LoVo cells showed shedding of the ectodomain, as detected by anti-(NC3 domain) and anti-Myc-tag antibodies in Western blotting of the precipitated media despite the furin-null background (Figure 3). Again, decRVKRCmk blocked the shedding (results not shown).

Time-dependence of ectodomain shedding

To determine the time-dependence of ectodomain release, shedding was characterized for 24 h, during which medium was recovered for precipitation, cells were lysed and the membrane fraction was separated from parallel cultures at given time points. Over this period, the cleaved ectodomain accumulated in the medium (Figure 4A) concomitantly with the growing expression of type XIII collagen in the cells, as seen in the Western blot of total cell lysates (Figure 4B). The levels of type XIII collagen and $\alpha 5 \beta 1$ integrin in the membrane fraction of the cells remained relatively constant throughout the experiment, with only a slight

Figure 3 Shedding of the ectodomain in transfected LoVo cells

Furin-deficient LoVo cells were transiently transfected with full-length type XIII collagen cDNA with a Myc-tag. The media containing the shed Myc-tagged ectodomain were precipitated, analysed by SDS/PAGE (8% gels), and detected with anti-(NC3 domain) (left-hand panel) and anti-Myc (right-hand panel) antibodies. The media reveal shedding of the Myc-tagged ectodomain despite the furin-null background of the LoVo cells. The cell lysates show strong expression of Myc-tagged full-length type XIII collagen resulting from transient transfection, as detected by the same antibodies. Detection of the untransfected cell lysate with anti-Myc antibody revealed a faint positive band representing endogenous Myc-positivity resulting from intrinsic expression of the Myc-epitope, characteristic for this cancer cell line. Size (in kDa) is indicated on the left. L, lysate; M, medium.

increase in the amount of membrane-bound type XIII collagen at the later time points (Figure 4B). In comparison, the relative increase in the amount of the membrane-bound type XIII collagen was smaller than the increase in the total cellular amount or that of the shed ectodomain in the medium. In line with this, in the analysis of shedding in fractions, the late-phase cultures released more ectodomain into the medium relative to the cell number than the new early-phase ones during the same time frame (Figure 4C). The increasing amount of shed ectodomain in the medium originated at least partly from cleavage at the plasma membrane, as the cell-surface-biotinylated HT-1080 cells showed a time-dependent pattern of ectodomain release (results not shown).

Perturbation of the Golgi reduces shedding of the ectodomain

As furin, PACE4 and PC7 of the PCs are located in the TGN [19,20,29], a malfunctioning Golgi apparatus may affect their function, and, consequently, the shedding of the ectodomain. BFA, monensin and A23187 were used to study this. BFA causes fusion of the Golgi saccular structure with the endoplasmic reticulum, whereas monensin disrupts vesicular transport within and from the TGN to the plasma membrane; i.e. both agents hamper the intracellular trafficking of maturing proteins. The calcium ionophore A23187 causes depletion of the intracellular calcium stores, rendering conditions for the calcium-dependent serine proteases suboptimal without substantially affecting intracellular trafficking [27]. The shedding assays were supplemented with 30 μ M BFA, 30 μ M monensin or 2 μ M A23187, followed by analysis of the precipitated media by Western blotting with an anti-(NC3 domain) antibody. Exposure for 6 h to all of these agents distinctly reduced shedding of the ectodomain (Figure 5).

Cells use various intracellular mechanisms to modulate shedding

To characterize the intracellular mechanisms involved in modulating the ectodomain shedding, cells were subjected to PMA and pervanadate, both recognized stimulators of shedding. PMA activates the intracellular PKC (protein kinase C) signalling cascade, and pervanadate inhibits the intracellular phosphotyrosine phosphatases [23,30,31]. PMA (50 ng/ml) stimulated release of the ectodomain in the HT-1080 cells, prominently so when the basal rate of shedding was low, as with adherent cells at early time points of 2–6 h (Figure 6A). At 24 h (results not shown) and in detached cells in suspension (Figure 6A), the enhancing effect of PMA was

Figure 4 Time-dependence of ectodomain shedding in HT-1080 cells

(A) The shed ectodomain in the precipitated media. (B) Uncleaved type XIII collagen and $\alpha 5$ integrin in the cell lysates and the membrane fraction of HT-1080 cells were analysed at time points of 0, 3, 6, 9, 12 and 24 h by SDS/PAGE (10% gels), followed by detection with anti-(NC3 domain) and anti-($\alpha 5$ integrin) antibodies. (C) Shedding of the ectodomain into the medium within 24 h was analysed in fractions consisting of 8-h periods (0–8 h, 8–16 h, 16–24 h). The results were normalized to the cell number of the cultures, counted at each time point, and are expressed as relative densitometric measurements. The densitometric values for the first time points in (B) and (C) were set at 100%. Actin in the cell lysates, as detected with an anti-actin antibody, served as a loading control in (B).

not so strong. In distinct contrast with the HT-1080 cells, PMA did not up-regulate ectodomain shedding in the SW-480 cells (Figure 6B). Pervanadate treatment, on the other hand, stimulated shedding in the SW-480 cells, but had an indeterminate effect in

Figure 5 Perturbation of the Golgi apparatus by BFA, A23187 and monensin

HT-1080 cell cultures were treated with 30 μ M BFA, 2 μ M A23187 and 30 μ M monensin for 6 h. Precipitated media were analysed by SDS/PAGE (10% gels), followed by detection with anti-(NC3 domain) antibody. The graph shows relative densitometric measurements of the Western blot bands of the precipitated media. The untreated control was set at 100%. Intact type XIII collagen and actin in the cell lysates were detected with anti-(NC3 domain) and anti-actin antibodies. Actin served as a loading control.

the HT-1080 cells (Figure 6C). Detachment of the cells from the substratum into suspension with 5 mM EDTA was found to lead to a notable up-regulation of ectodomain shedding, regardless of the cell line (Figures 6A and 6B). DecRVKRCmk markedly reduced ectodomain release in both adherent and detached cells, again irrespective of the cell line (Figures 6A and 6B).

Functional characterization of the shed ectodomain

The biological relevance of the soluble ectodomain was addressed by studying its effect on cell adhesion, migration and proliferation. The ectodomain had no apparent influence on the adherence of primary fibroblasts during prolonged adhesion, but in a short-term experiment reflecting the immediate initial stages of adherence, it reduced cell adhesion to a statistically significant extent only on a vitronectin surface, while leaving cell adhesion on fibronectin, EHS laminin, poly-D-lysine, type I or IV collagens unaffected (results not shown).

The effect of the ectodomain on cell migration was examined with two experimental set-ups. In a wound closure assay (Figure 7), the denuded space between the edges of the 'wound' had been covered more efficiently at 24 h by migrating HT-1080 cells in a serum-free medium devoid of ectodomain than by those in a CM enriched in the shed ectodomain (Figures 7A and 7B). When the cells were then subjected to exogenously supplemented ectodomain, this reduced the motility of the HT-1080 cells as compared with untreated cells in a similar fashion to the CM treatment (Figure 7C), implicating the ectodomain as a factor contributing to the effect. The ectodomain did not cause an overall block in cell motility, but instead permitted a front of diffuse, yet clearly hampered, cell migration. As the cells exhibited a relatively typical migratory phenotype, it is likely that the ectodomain rendered the conditions suboptimal, although not entirely intolerable, for cell migration. Figures 7(D)–7(F) show

the early stage of migration at 4 h, at which time the cells show equal initial migratory activity despite the treatment. In the haptotactic transmigration assay on isolated substrata, exogenously added ectodomain interfered with the motility of the primary fibroblasts specifically on vitronectin (results not shown).

The ectodomain compromised the rate of cell growth when present in normal culture medium containing serum. The number of cells in the control culture had doubled within 48 h, whereas that in the ectodomain-treated culture showed only a marginal increase. Also, the ectodomain-supplemented culture exhibited an almost equal rate of cell proliferation to that of the control culture at 72 h (Figure 8A). As fibronectin and vitronectin are the major cell-attachment factors in serum [32], the experiment was repeated under serum-free conditions on these isolated substrata, whereupon ectodomain supplementation did indeed inhibit cell proliferation specifically on the vitronectin surface (Figure 8B), leaving cell growth on fibronectin unaffected (Figure 8C). Morphological analysis of the cells revealed that the ectodomain supplementation prevented the early phase of cell adherence and spreading. At 4 h, the ectodomain-treated cells exhibited an aberrant morphology with inadequate spreading as compared with the BSA-treated control cells. The round cells displayed total lack of spreading, while the others showed preliminary spreading, but none of them were of the characteristic fibroblast-like initial phenotype like the control cells (Figures 8D and 8E). The morphological differences were not evident in a continued incubation for 24 h, at which time the cells in both cultures showed a fully extended fibroblast-like morphology (Figures 8F and 8G).

As a substratum, the ectodomain provided poor support for cell adhesion and migration. In a prolonged adhesion experiment of 3 h, primary fibroblasts showed little adherence to the ectodomain relative to other substrata, such as vitronectin, fibronectin, EHS laminin, and collagen types I and IV (Figure 9A). The cells in the haptotactic transmigration assay in Boyden chambers also migrated poorly on the ectodomain as compared with the other surfaces tested (Figure 9B). Morphologically, the few cells that had migrated showed insufficient spreading on the ectodomain (results not shown), as observed above.

DISCUSSION

The transmembrane collagen types XIII, XVII, XXIII and XXV are currently recognized as shedding their ectodomains from the plasma membrane [10–13]. These soluble ectodomains have exhibited biological activity by interacting with a number of ECM proteins *in vitro*, for example, the ectodomain of type XIII collagen with fibronectin, nidogen-2 and perlecan, that of type XXIII collagen with heparin, and that of type XXV collagen with the fibrillar β -amyloid peptide [12,13,22]. In view of such characteristics, it was of interest for us to explore the shedding of the type XIII collagen ectodomain in mammalian cells more closely and to consider its biological relevance *in vivo*.

The type XIII collagen ectodomain was released in a uniform size by several mammalian cell types regardless of whether they were of tumour or non-tumour origin, suggesting a universal cleavage site. The shed ectodomain retained its integrity, as it was not subjected to immediate degradation after its release. The actual size of the cleaved ectodomain corresponded well to earlier results with the insect cells [11], and to size estimates calculated from the amino acid sequence, on the assumption that the shedding occurs at the consensus cleavage site for PCs juxtaposed to the transmembrane region. Various mammalian cell types were found to employ different intracellular signalling mechanisms to

Figure 6 Effect of PMA, cell detachment and pervanadate on shedding

Adherent and EDTA-detached (**A**) HT-1080 (2 h, 6 h) and (**B**) SW-480 cells (2 h) were treated with 50 ng/ml PMA and 80 μ g/ml decRVKrcmk (cmk) for the time periods indicated. (**C**) The effect of pervanadate was evaluated in SW-480 and HT-1080 cells, by treating the adherent cells with 10 or 25 μ M pervanadate for 6 h. The precipitated media were analysed by SDS/PAGE (10% gels), followed by detection with anti-(NC3 domain) antibody. The histograms show relative densitometric measurements of the Western blot bands of the precipitated media, with the untreated adherent control cells set at 100% in (**A**) and (**B**). The untreated controls for the SW-480 and HT-1080 cells were set at 100% in (**C**). Intact type XIII collagen or actin in the cell lysates were detected with anti-(NC3 domain) or anti-actin antibodies. Actin served as a loading control.

augment shedding, e.g. activation of the PKC signalling cascade or inactivation of the phosphotyrosine phosphatases. Regardless of the mechanism, the shedding process was mechanistically similar in all cases.

The furin family of the mammalian Kex/subtilisin-like PC group was implicated as being solely responsible for this shedding, since cleavage was effectively blocked only by decRVKrcmk, a specific inhibitor of this group [33]. In the present study, we have shown shedding of type XIII collagen ectodomain in a number of mammalian cells known to express at least furin of the PCs [18]. Additionally, the transfected furin-null LoVo cells expressing only PACE4 and PC7 [27,28] showed shedding of the ectodomain. Furin, PC7 and PACE4 share an overlapping substrate and sequence specificity, and a close cellular localization

[18–21], due to which characteristics any one of the three could be implied in the ectodomain shedding. The blocking effect of decRVKrcmk on LoVo cells is explained by the lack of strict furin specificity [20]. Shedding is generally considered to take place at the cell surface, mainly through the action of MMPs or ADAMs [2,4–6], but the lack of effect of all the other inhibitors tested argues against the involvement of MMPs, ADAMs, the cysteine and aspartate proteases, chymotrypsin, trypsin, aminopeptidases, metalloendopeptidases or the uPA (urokinase plasminogen activator)/plasmin-driven activation of MMPs or active plasmin [34] in the type XIII collagen ectodomain shedding. In this respect, type XIII collagen differs from type XVII collagen [15].

Perturbation of vesicular trafficking within and from the Golgi apparatus by BFA and monensin, or suboptimal intracellular

Figure 7 Effect of the ectodomain on the migration of HT-1080 cells

In the wound closure assay, scratch wounds were created in a HT-1080 cell culture with the tip of a pipette. After washing, the cells were supplemented with serum-free culture medium (**A** and **D**), serum-free CM of HT-1080 cells (**B** and **E**), or serum-free medium with 0.5 μ M purified ectodomain (**C** and **F**), and permitted to migrate from the edges of the wound over the denuded area at +37 °C for 24 h (**A–C**) or 4 h (**D–F**). At both time points, the non-fixed cells were photographed with phase-contrast optics. The influence of the CM or exogenous ectodomain on cell motility was evaluated by comparing the migration of cells into the denuded space between the treated and non-treated cultures. Scale bar, 100 μ m.

Ca²⁺ levels brought about by A23187 [27,29], resulted in clearly reduced shedding of the ectodomain. Based on this and our inhibitor data, we thus conclude that the TGN, possessing these calcium-dependent proteases, is an important intracellular processing site for type XIII collagen. This concept was also supported by the PMA-derived enhancement of ectodomain shedding in HT-1080 cells, in that PMA acts via PKC and casein kinase II to mediate the phosphorylation of furin, which anchors it to the TGN [28]. Although concentrated in the Golgi apparatus, furin, PACE4 and PC7 are recycled through a regulated trafficking route between the TGN, endosomal compartments and the cell surface [18,20]. As the cell-surface-biotinylated cells displayed release of ectodomain, with abolition by decRVKRCmk, we also postulate that the plasma membrane can also be used by these PCs to excise the ectodomain. The time-dependent follow-up of ectodomain shedding suggested that cells strive to maintain an equilibrium in type XIII collagen content on the plasma membrane despite growing cellular amounts, and this was accomplished at least in part by effective cleavage at the cell membrane. We do not know at the moment, however, whether either of the cleavage locations represents a site of physiological, controlled and timely response to a given stimulus experienced by a cell at a given time, or merely a site for unloading type XIII collagen that has outlived its purpose. Divergent possibilities evolve out of the findings, namely that the full-length type XIII collagen may represent a precursor of the cleaved functional type XIII collagen ectodomain, or alternatively, type XIII collagen may show a dualistic phenotype with membrane-bound and soluble isoforms, both functioning actively under specific conditions. The

Figure 8 Effect of the ectodomain on the proliferation of primary fibroblasts

In the proliferation assay, 5×10^3 fibroblasts were applied in triplicate to the wells of a 96-well plate in a medium containing serum without any coating of the wells (**A**), or on to vitronectin (**B**) or fibronectin (**C**) surfaces in a special serum-free medium (Fibroblast Growth Medium; PromoCell). The cells were supplemented with 0.5 μ M purified ectodomain (COLXIII EC) or BSA and allowed to grow for 4, 24, 48 and 72 h, with daily renewals of the medium. Cell proliferation at each time point was measured by assaying the DNA content of the cells adhering to the wells and presented relative to that at 4 h, which was set at 1.0. The graphs are means $\pm$ S.D. for three individual experiments. Statistical significance was determined with Student's *t* test: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. The cells were photographed under a phase-contrast microscope at 4 h (**D** and **E**) and 24 h (**F** and **G**) during the growth assay. The cells were grown in a medium containing serum supplemented with BSA (**D** and **F**) or 0.5 μ M ectodomain (COLXIII EC) (**E** and **G**). The round cells (indicated with arrowheads) displayed a lack of spreading, while some cells showed preliminary spreading (indicated with arrows) in (**E**). Scale bar, 25 μ m.

latter option is supported by the universal localization of the full-length type XIII collagen in various adhesive structures of cells and tissues [17,35] in a matrix-independent manner (T. Väisänen, unpublished work).

The fact that detachment of cells from the underlying substrata by EDTA up-regulated ectodomain shedding merits special attention, as cell detachment could represent a regulated, carefully

Figure 9 The ectodomain as a substrate for cell adhesion and migration

(A) Fibroblasts (3×10^4) were plated in triplicate in serum-free medium on the wells of a 96-well plate coated with BSA, vitronectin (Vn), fibronectin (Fn), EHS laminin (Ln), collagens type I (COLI) and IV (COLIV) or purified ectodomain of type XIII collagen (COLXIIIEC). After adherence for 3 h, the number of adherent cells was determined by quantifying the DNA content of the cells adhering to the wells. The results are expressed as means of emissions derived from three separate experiments. (B) Fibroblasts (5×10^4) were plated in serum-free medium on Boyden chambers coated with BSA, fibronectin, EHS laminin, type I collagen or type XIII collagen ectodomain. The cells were allowed to migrate for 3 h, after which the cells that had migrated were counted from six randomly selected fields per membrane. The results are expressed as means $\pm$ S.D. of the number of cells migrating.

timed stimulus of the kind postulated above, e.g. during repeated cell attachment–detachment cycles in cell migration and proliferation. Such a scenario has been presented for the neural adhesion protein L1 [30,31]. The cleaved type XVII collagen ectodomain is also known to affect keratinocyte motility [15].

In view of the known location of intact type XIII collagen [17,35], the potential biological context of the shed ectodomain may be related to adhesive-dependent cell functions. The *in vitro* interaction of the ectodomain with ECM proteins [22] makes it feasible that the released ectodomain may be retained by the ECM *in vivo*, which could in turn alter the biological properties of the matrix proteins, either due to direct binding or by steric hindrance of interaction with cell-surface receptors, such as integrins. The effects of the shed ectodomain were anti-adhesive, anti-migratory and anti-proliferative on a vitronectin surface, but not on fibronectin. Vitronectin is one of the major proteins mediating cell adhesion in cultures containing serum [32,36], and is recognized as supporting cell adhesion, spreading, migration and proliferation [37,38]. Both proteins contain collagen-binding domains and an RGD (Arg-Gly-Lys)/cell-adhesion domain, which flank one another in vitronectin, but lie further apart in fibronectin [36,39]. Cells expressing vitronectin plasma-membrane receptors of the integrin α_v subfamily attach to vitronectin via the RGD sequence [40,41]. Consequently, steric masking of the RGD/cell-adhesion

domain by ectodomain association to the juxtaposed collagen-binding domain might be the mechanism for the observed effect, by reducing the availability of the necessary integrin binding sites, hence eventually preventing integrin-mediated cell adhesion. This would subsequently affect the rates of cell migration and proliferation on vitronectin, because cell migration involves transient, integrin-mediated adhesion of cells, and flawed cell adherence is known to be reflected in cell migration [42,43]. The time window for the ectodomain influence was apparently a narrow one, since the effects were notable at the very initial stages in cell attachment, at which time the cells also exhibited defective spreading.

The ectodomain as a substratum proved to afford little support for cell adhesion and migration, as the primary fibroblasts showed very little adherence or migration on it as compared with fibronectin, vitronectin or collagen types I and IV, for example. The difference relative to the last two is noteworthy, since dermal fibroblasts are known to express the collagen receptors integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [44]. This implies that cell adhesion to type XIII collagen is not mediated by these integrins. CHO (Chinese-hamster ovary) cells overexpressing integrin $\alpha 1\beta 1$ have been reported to adhere to recombinant ectodomain, albeit with imperfect cell spreading, suggesting impaired cell adhesion [45]. The ectodomain of type XVII collagen has shown a similar effect of decreased cell motility, although otherwise its functional ramifications have yet to be established [15].

It is considered a paradigm that adherent cells share a bidirectional flow of spatial and temporal information with the surrounding ECM. This reciprocal communication provides cells with developmental and/or function-modulating signalling cues, and reciprocally, reorganizes the local ECM through differential cellular expression of the matrix components [46,47]. We have shown that the soluble type XIII collagen ectodomain can modulate cell behaviour on a specific substratum, here vitronectin. Should the ectodomain be released in vitronectin-rich surroundings, the association between the two may alter the cell-binding qualities of vitronectin, resulting in cells with less capability for adherence. In cancer with locally accumulated vitronectin [48], reduced cell adherence to vitronectin could enhance the metastatic capacity of the tumour cells [49]. The findings that collagens, including transmembrane type XIII collagen, release either non-collagenous or extracellular domains into the pericellular ECM [10–12,50] not only conform well to the established concept of ongoing exchange of information between cells and the ECM, but imply that collagens may also serve in far more active regulatory roles than their hitherto recognized structural functions.

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