Furin interacts with proMT1-MMP and integrin αV at specialized domains of renal cell plasma membrane

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Summary

Matrix metalloproteinases (MMPs) and integrins are essential for cell and extracellular matrix homeostasis. Both membrane type-1 MMP (MT1-MMP) and the integrin αV subunit are fully activated upon cleavage at a furin recognition site. Furin is shuttled to the cell surface through the trans-Golgi network and endosomal system, and its only known role on plasma membrane consists in activation of opportunistic pathogenic entities. Here, we report findings about the interaction of furin with MT1-MMP and the integrin αV at the cell surface. By using in vivo gene delivery, western blotting and immunogold electron microscopy, we provide evidence of significant pools of furin and proMT1-MMP along the surface of cells lining basement membranes. Moreover, furin and integrin αV are frequently found associated with the slit diaphragm of renal podocytes and around endothelial fenestrations. ProMT1-MMP, by contrast, is concentrated at the slit diaphragm. Coimmunoprecipitations and double immunogold labelings indicate that furin interacts with proMT1-MMP and αV at points of insertion of the slit diaphragm. Our results suggest that these focalized complexes could trigger basement membrane proteolysis either directly by activation of proMT1-MMP or indirectly by promoting activation of proMMP2.

Key words: Furin, ProMT1-MMP, Integrin, Kidney glomerulus, Cell surface

Introduction

Synergistic interactions of cellular and extracellular elements are needed to establish and maintain multicellular organization and tissue-specific functions. A simple yet functionally complex example of such cooperation is encountered in the renal glomerulus. The glomerular wall, the highly specialized unit responsible for the selective ultrafiltration of the blood, is composed of three layers: a fenestrated endothelium, the glomerular basement membrane (GBM) and the filtration slits formed by the interdigitating foot processes of the visceral epithelial cells (podocytes). The slit diaphragm bridges the filtration slit to form specialized intercellular junctions (Kanwar and Venkatachalam, 1992). The structural integrity of the foot processes and their slit diaphragms with the appropriate turnover of the GBM components are key elements for the proper function of the glomerulus. A plethora of nephropathies are associated with podocyte-foot-process fusion and detachment, proteolytic degradation of the GBM, and extracellular matrix (ECM) accumulation, resulting in the loss of essential plasma proteins into the urinary space (Lenz et al., 2000; Barisoni and Kopp, 2002). Knowledge of the interactions among cell surface proteins mediating GBM turnover is crucial for an understanding of glomerulus function.

Furin is a calcium-dependent serine protease of the subtilisin-like proprotein convertase family. This type I transmembrane glycoprotein is involved in pro-domain cleavage of many bioactive proteins traveling along the constitutive secretory route. It cleaves proteins at the C-terminal side of multibasic amino acid motifs such as R-X-K/R-R and R-X-X-R (Molloy et al., 1999). Knockout of furin gene is embryonic lethal (Roebroeck et al., 1998). This underscores the importance of this protease in the maturation, function and activation of several hormones, growth factors and cell surface receptors (Molloy et al., 1999). Furin is also efficient at processing several adhesion-related proteins. Furin processes and activates proproteins involved in cell-ECM and cell-cell interactions such as pro-α-integrin subunits and pro-cadherins (Lehmann et al., 1996; Posthaus et al., 1998; Lissitzky et al., 2000). In addition, furin cleaves protein substrates such as pro-membrane-type-1 matrix metalloproteinases (proMT1-MMP) and pro-transforming-growth-factor β, which play major roles in the regulation of the ECM and basement membranes (Dubois et al., 1995; Pei and Weiss, 1996). Incidentally, these adhesion and ECM-related proteins are important for the structure and function of the glomerulus. Thus, by activating these proteins, furin could play pivotal roles in pathophysiological processes. Although significant information has been gathered using cell culture, little is known about the trafficking and function of furin in situ. Moreover, the physiological function of furin at the cell surface remains unknown.

The plasma-membrane-anchored matrix metalloproteinase (MMP) MT1-MMP is involved in many important tissue-remodeling events because of its ability to degrade ECM proteins either directly or indirectly by activating downstream soluble MMPs such as the proMMP2 (Sternlicht and Werb, 2001). MMP2, which is essential for glomerulogenesis (Serluca et al., 2002), degrades the major constituent of

basement membranes, type-IV collagen, and is involved in various glomerular diseases (Lenz et al., 2000). In addition, MT1-MMP and MMP2 can degrade several other components of the GBM (Sternlicht and Werb, 2001). MT1-MMP is synthesized as a proenzyme with two potential furin cleavage sites at the end of the propeptide domain. Yana and Weiss (Yana and Weiss, 2000) have recently demonstrated that the removal of the pro-domain is a prerequisite for MT1-MMP to acquire catalytic activity and that furin is probably its major processing enzyme. Although it has been demonstrated that full-length proMT1-MMP is the prominent form appearing at the plasma membrane of many cell types, the subcellular site of activation and the identity of the activating enzyme remain controversial (Sato et al., 1994; Okumura et al., 1997; Cao et al., 1998; Lehti et al., 1998; Sternlicht and Werb, 2001). Nonetheless, a proprotein-convertase/MT1-MMP/MMP2 axis has been proposed to conduct ECM remodeling (Yana and Weiss, 2000; de Kleijn et al., 2001).

In the present study, we report several novel findings that could shed some light on a furin/MT1-MMP activation cascade operating at the renal glomeruli. By using in vivo gene delivery, western blotting and immunogold electron microscopy (EM), we provide evidence of significant pools of furin and proMT1-MMP in glomeruli, concentrated along the basal surface of epithelial and endothelial cells, lining the GBM. Furin was mainly found at the slit diaphragm of podocytes and close to endothelial fenestrations, whereas proMT1-MMP is concentrated at the slit diaphragm. Coimmunoprecipitation experiments on isolated membrane fractions of glomerular cells indicate that both enzymes are interacting and double immunogold labelings revealed that furin and proMT1-MMP are colocalized on plasma membranes, particularly at the points of insertion of the slit diaphragms. Moreover, we show that cell surface furin interacts with the αV integrin subunit, which participates in the activation of proMMP2 (Deryugina et al., 2001). These results suggest that the interaction of furin with these proteins could trigger GBM degradation either directly, by the activation of cell surface proMT1-MMP, or indirectly, by promoting the activation of proMMP2. Moreover, the very peculiar localization pattern of furin and proMT1-MMP suggests that a mechanism of focalized pericellular proteolysis is involved in GBM turnover and must play an important role in the maintenance of the specialized structures of the glomerular wall.

Materials and Methods

Antibodies and reagents

The rabbit anti-furin-N-terminus antibody (raised against amino acids 187-198 of furin) and the mouse monoclonal anti-furin-N-terminus antibody were from Alexis Biochemicals. The goat and rabbit anti-furin-C-terminus antibodies were from Santa-Cruz Biotechnology and Affinity Bioreagents, respectively. The rabbit anti-hemagglutinin (HA) tag, the rabbit anti-MT1-MMP proregion, the FITC-conjugated anti-rabbit IgG antibodies and the protease inhibitor cocktail were from Sigma. The rabbit anti-integrin-αV-C-terminus and anti-β3 antibodies were from Chemicon. Plasmid pSVLfur, containing the human furin gene (nucleotides 101-4180), was from the American Type Culture Collection. Restriction enzymes were from New England Biolabs. Bicinchoninic acid protein assay reagent kit and dithio-*bis*(succinimidylpropionate) (DSP) were from Pierce Chemical. The Lumi-Light Plus chemiluminescence western blotting detection kit was from Roche Diagnostics. The human recombinant furin enzyme was from Affinity Bioreagents. Protein-A/gold conjugates (5 nm and 10 nm) were prepared as described by Bendayan (Bendayan, 1995).

DNA manipulations and plasmid constructions

From the plasmid pSVLfur, the complete coding sequence of furin was isolated, modified and amplified by PCR using chemically synthesized oligonucleotides specific for furin (Life Technologies). Oligonucleotide primers were designed to introduce restriction sites in the furin sequence to subclone only the coding sequence (nucleotides 217-2601) of the gene into the mammalian expression vector pCDNA3/RSV (Jockers et al., 1996). The forward primer 5′-AGCCACCTGTCCCCC*AAGCTT*A<u>C</u>CC**ATG**GAGCTGAG-3′ allowed the introduction of a *Hin*dIII restriction site (italics) upstream of the start codon (in bold). An adenine (underlined) was added to complete an optimal sequence (ACCATGG) for initiation of translation by eukaryotic ribosomes (Kozak, 1986). The reverse primer 5′-GGTGGGCAGTGGGCTCA*TCTAGA***TCA**T<u>GGCGCGCC</u>GAGGG-CGCTCTGGTCTTT-3′ allowed the introduction of an *Xba*I site (italics) immediately downstream of the furin translational stop codon (in bold). An *Asc*I restriction site (underlined) was introduced upstream the stop codon to allow the insertion of an HA-tag coding sequence. A thymine was also added immediately after the *Asc*I site to keep the good reading frame.

Following the PCR, the amplified 2.4 kb furin cDNA fragment was digested with *Hin*dIII and *Xba*I restriction enzymes, and ligated into the multiple cloning site of the vector pCDNA3/RSV resulting in pCDNA3/RSV/Furin. The pCDNA3/RSV/Furin-HA vector was constructed by ligating annealed oligonucleotides coding for the HA-tag peptide (Lesage et al., 2000) into *Asc*I-digested pCDNA3/RSV/Furin vector. The resulting construct encoded two copies of the HA epitope in the C-terminus tail of the furin protein.

In vivo gene delivery

Furin and furin-HA-encoding vectors were introduced into mouse tissues using the TransIT in vivo gene delivery system according to the manufacturer's instructions (Mirus). Briefly, 15 μl of 1 mg ml^{-1} pCDNA3/RSV/Furin or pCDNA3/RSV/Furin-HA were mixed with 15 μl of a cationic polymer solution and 170 μl sterile endotoxin-free water. The resulting cationic DNA particles of less than 100 nm were added to a 2 ml delivery solution and the entire volume was then injected through a 27-gauge needle into the tail vein of an 18 g immunodeficient SCID mouse, in 6 seconds. For each of the furin and furin-HA vectors, seven mice were injected. In control experiments, mice were injected with the plasmid pCDNA3/RSV without furin cDNA. 24 hours after gene delivery, animals were anesthetized and tissues were sampled and processed for biochemical and immunocytochemical assays.

Tissue preparation

Kidneys from control 150 g male Sprague Dawley rats and from control and plasmid-injected SCID mice were removed, divided into three parts and processed as follows. One part was frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound. 5-μm-thick cryosections were mounted on glass slides, fixed in acetone:ethanol (1:1) at –20°C and washed in PBS at room temperature. A second part of the tissue was cut into 1 mm^3 pieces, fixed by immersion in 4% paraformaldehyde-lysine-periodate, embedded at low temperature in Lowicryl K4M and cut into semi-thin (0.5 μm) and ultrathin (100 nm) sections (Bendayan, 1995). Semi-thin sections were mounted on glass slides and the ultrathin ones on Parlodion-carbon-coated nickel grids. The last part was frozen in liquid nitrogen and kept at –80°C for biochemical assays.

Immunofluorescence

Fixed cryosections were incubated with the rabbit anti-furin-N-terminus antibody or the rabbit anti-HA antibody overnight at 4°C followed by the FITC-conjugated goat anti-rabbit IgG for 1 hour. Sections were examined with a Leitz Orthoplan DM RB fluorescent microscope. Control experiments, omitting the primary antibody, incubation with normal sera and adsorption with corresponding antigens were performed for each labeling protocol.

Immunogold labeling experiments

The labeling procedure was carried out as previously described (Bendayan, 1995). Ultrathin sections were incubated overnight at 4°C with the appropriate primary antibody followed by protein A-gold (5 nm or 10 nm; OD_{525}=0.5) for 30 minutes. The tissue sections were observed with a Philips 410SL electron microscope.

Double labeling experiments were conducted on both sides of ultrathin sections mounted on uncoated nickel grids (Bendayan, 1995). The labelings were carried out essentially as described above using the anti-proMT1-MMP or the anti-integrin αV antibodies on one side of the grids and the rabbit anti-furin-N-terminus antibody on the other side of the grids. ProMT1-MMP and integrin antigenic sites were detected with protein-A/5-nm-gold complex, and furin with protein-A/10-nm-gold complex. Quantitative evaluations of the double labelings were conducted as previously described (Bendayan, 1995).

Glomerulus isolation, fractionation and lysis

Glomeruli were isolated from freshly dissected rat renal cortex by sequential sieving as previously described (Regoli and Bendayan, 1999). Intact isolated glomeruli were suspended in modified RIPA buffer (50 mM PBS, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 200 μg ml^{-1} PMSF and 1× protease inhibitor cocktail), homogenized and centrifuged for 20 minutes at 14,000 ***g***. The supernatants were used for biochemical analysis.

Fractionation of isolated glomeruli was conducted in accordance to the modified protocol of Kerjaschki et al. (Kerjaschki et al., 1989). Glomeruli were suspended in 200 mM Na_2CO_3, pH 11, containing a protease inhibitor cocktail, homogenized and submitted to sonication for 5 minutes in a Bransonic ultrasonic bath. The homogenate was then separated into an insoluble pellet, containing mainly GBM and cell debris, and a supernatant by centrifugation for 5 minutes at 2000 ***g*.** The supernatant was resolved into a total membrane pellet and a soluble supernatant by centrifugation for 60 minutes at 100,000 ***g*** in a Beckman XL-70 ultracentrifuge using a SW-60Ti rotor. The total membrane pellet was resuspended in ice-cold modified RIPA buffer, homogenized and kept at 4°C for 60 minutes with end-over-end rotation. The homogenate was centrifuged for 20 minutes at 14,000 ***g*** and the resulting supernatant was stored at –80°C until needed for biochemical assays.

Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as a standard.

Chemical crosslinking

In order to detect unstable protein-protein interactions, total glomerular membrane fractions were crosslinked with the thiol-cleavable reagent DSP according to the protocol of Löster et al. (Löster et al., 1995). Briefly, the solubilized material (500 μl) was mixed with 12.5 μl of DSP, from a freshly made 25 mM stock in dimethyl sulfoxide, and allowed to react for 15 minutes at room temperature. The crosslinker was then quenched by adding 50 μl of 100 mM Tris-HCl, pH 8.0, and the resulting fractions were used for coimmunoprecipitation studies as described below.

Digestion of glomerular membranes by soluble recombinant furin

The experimental conditions for the assessment of proMT1-MMP cleavage by purified soluble furin were adapted from a previous report (Seger and Shaltiel, 2000). Briefly, a fraction of solubilized glomerular membranes (50 μg) was incubated with recombinant furin (5 U) in 25 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 1 mM $CaCl_2$ and 1 mM β-mercaptoethanol for 4 hours at 37°C. Reducing sample buffer was then added and membrane proteins were separated by SDS-PAGE and analyzed by western blotting.

Immunoprecipitation

Immunoprecipitations were conducted as previously described (Arias et al., 2000). The detergent lysates, precleared by incubations with preimmune rabbit serum and protein-A/Sepharose, were incubated either with 5 μl of the rabbit anti-proMT1-MMP or 5 μl of the rabbit anti-integrin-αV antibody for overnight at 4°C under agitation. The immune complexes were recovered by the addition of protein-A/Sepharose and washed with the modified RIPA buffer. The immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting.

Western blot analysis

Protein samples were boiled for 4 minutes in reducing SDS-sample buffer, separated by SDS-PAGE on 7.5% or 10% Ready gels (Bio-Rad) or handmade polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes. Immunodetection of proteins on nitrocellulose membrane was conducted as follows. Briefly, the membranes were blocked with TBS containing 0.05% Tween 20 and 1% skimmed milk, and incubated overnight at 4°C with the appropriate antibodies. Bound antibodies were then revealed with the Lumi-Light Plus chemiluminescence detection kit. The results were recorded by exposition of the membranes to Kodak X-Omat-AR films.

Results

Localization of endogenous and overexpressed furin in kidney glomeruli

Furin is expressed at low levels and its localization in cells is often extrapolated from studies using overexpression systems ascribing it almost exclusively as a trans-Golgi network (TGN) resident (Bosshart et al., 1994). Therefore, our first goal was to establish the location of endogenous furin by means of sensitive morpho-cytochemical approaches. Immunofluorescence performed on frozen normal rat renal tissue revealed furin in detectable amounts in glomerular cells (Fig. 1A). The positive signal was located close to nuclei and along the basolateral membranes. In accordance with this, the immunoblot analysis of electrophoresed proteins from solubilized isolated glomeruli revealed a single band at ~98 kDa, the molecular weight of mature rat furin (Fig. 1F) (Wouters et al., 1998).

In order to identify the precise subcellular location of endogenous furin in glomerular cells, immunogold was applied at the EM level. As expected, the Golgi apparatus of glomerular cells, particularly podocytes, was labeled (Fig. 1B). The endoplasmic reticulum was weakly labeled whereas very few gold particles were located over nuclei and mitochondria (Fig. 1B). The novel unexpected result concerned the labeling obtained at the surface of the glomerular cells. Indeed, furin immunolabeling, as revealed by gold particles, was present at both the lumenal and ablumenal plasma membranes of glomerular endothelial cells (Fig. 1C). Strikingly, the gold particles were very frequently associated with the endothelial fenestrations (Fig. 1C,D).

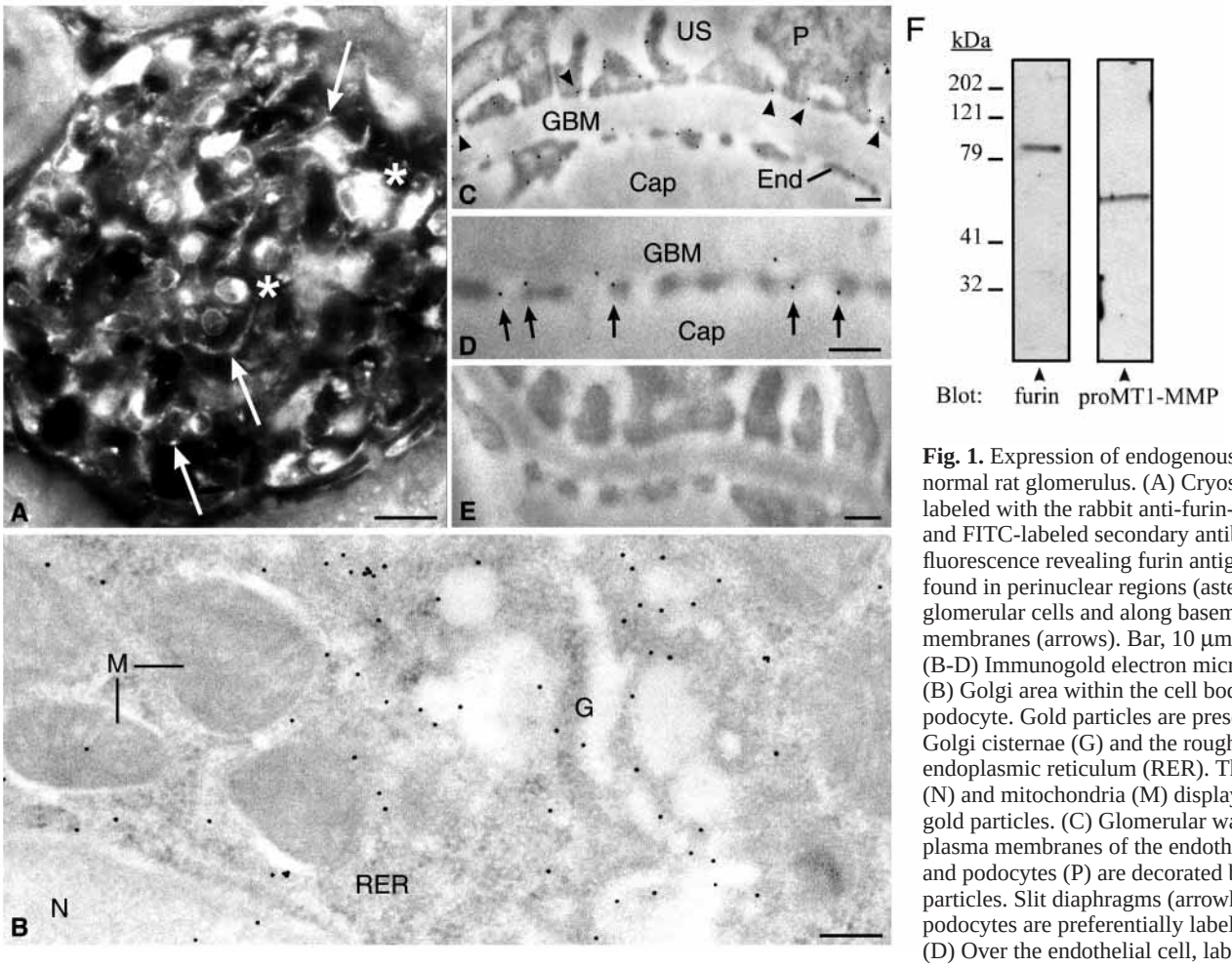

Fig. 1. Expression of endogenous furin in normal rat glomerulus. (A) Cryosection labeled with the rabbit anti-furin-N-terminus and FITC-labeled secondary antibodies. The fluorescence revealing furin antigenic sites is found in perinuclear regions (asterisks) of glomerular cells and along basement membranes (arrows). Bar, 10 μm. (B-D) Immunogold electron microscopy. (B) Golgi area within the cell body of a podocyte. Gold particles are present over the Golgi cisternae (G) and the rough endoplasmic reticulum (RER). The nucleus (N) and mitochondria (M) display very few gold particles. (C) Glomerular wall. The plasma membranes of the endothelium (End) and podocytes (P) are decorated by gold particles. Slit diaphragms (arrowheads) of podocytes are preferentially labeled. (D) Over the endothelial cell, labeling for furin is particularly focalized around the fenestrations (arrows). (E) Control conditions. The anti-furin antibody was preadsorbed with an excess of its antigen. The glomerular wall is exempt of gold particles. (B-E) Bars, 200 nm. Abbreviations: Cap, capillary lumen; GBM, glomerular basement membrane; US, urinary space. (F) Glomeruli homogenates were resolved by 10% SDS-PAGE and proteins were electrotransferred onto nitrocellulose membranes. The rabbit anti-furin-N-terminus and anti-proMT1-MMP antibodies were used for immunoblotting and were revealed by chemiluminescence. Single bands were obtained for furin (~98 kDa) and proMT1-MMP (~63 kDa).

Moreover, furin was associated to podocytes, particularly at the slit diaphragms and, to a lesser extent, at apical and basal membrane domains facing the urinary space and the GBM, respectively (Fig. 1C). Control experiments performed by adsorbing the antibody resulted in a major reduction of the labelings (Fig. 1E). The use of either one of the four anti-furin antibodies generated similar results.

The immunodetection of endogenous furin at the cell surface raises concern about furin being an enzyme confined to the TGN as demonstrated in overexpressing cell systems. Overexpression might lead to artefactual distribution by saturating retention or retrieval mechanisms (Wouters et al., 1998). In order to compare the localization of the normally expressed endogenous and the overexpressed furin in vivo, we used a gene delivery system that, upon tail-vein injection of DNA-polymer complexes in mouse, leads to high levels of transgene expression in multiple organs. We have analysed furin expression in the kidney of mice 24 hours after the injection of the plasmid pCDNA3/RSV/Furin or pCDNA3/RSV/Furin-HA. By immunofluorescence, cells expressing high levels of furin were found in glomeruli (Fig. 2A,B). In the pCDNA3/RSV/Furin-injected mice, some renal cells showed normal staining, whereas others displayed very intense signals, probably caused by overexpression of furin (Fig. 2A). Staining of perinuclear regions, vesicular structures and plasma membranes were similar, although of higher intensity, to those obtained for endogenous normally expressed furin (Fig. 1). Obviously, these signals include those of endogenous as well as overexpressed furin (Fig. 2A). In order to discriminate and to reveal solely the overexpressed furin, we screened the pCDNA3/RSV/Furin-HA injected mice. The expression of the HA-tagged furin in glomerular cells was clearly revealed by immunofluorescence (Fig. 2B). Only few cells expressed furin-HA, a result expected in tissues of tail-vein plasmid-DNA-injected mice (Budker et al., 1996). Perinuclear, vesicular and cell surface labelings similar to those described above were obtained (Fig. 2B).

The subcellular localization of overexpressed furin was carried out at the EM level. Furin- and furin-HA-transduced cells were identified on semi-thin sections and consecutive ultrathin sections were submitted to the immunogold labeling.

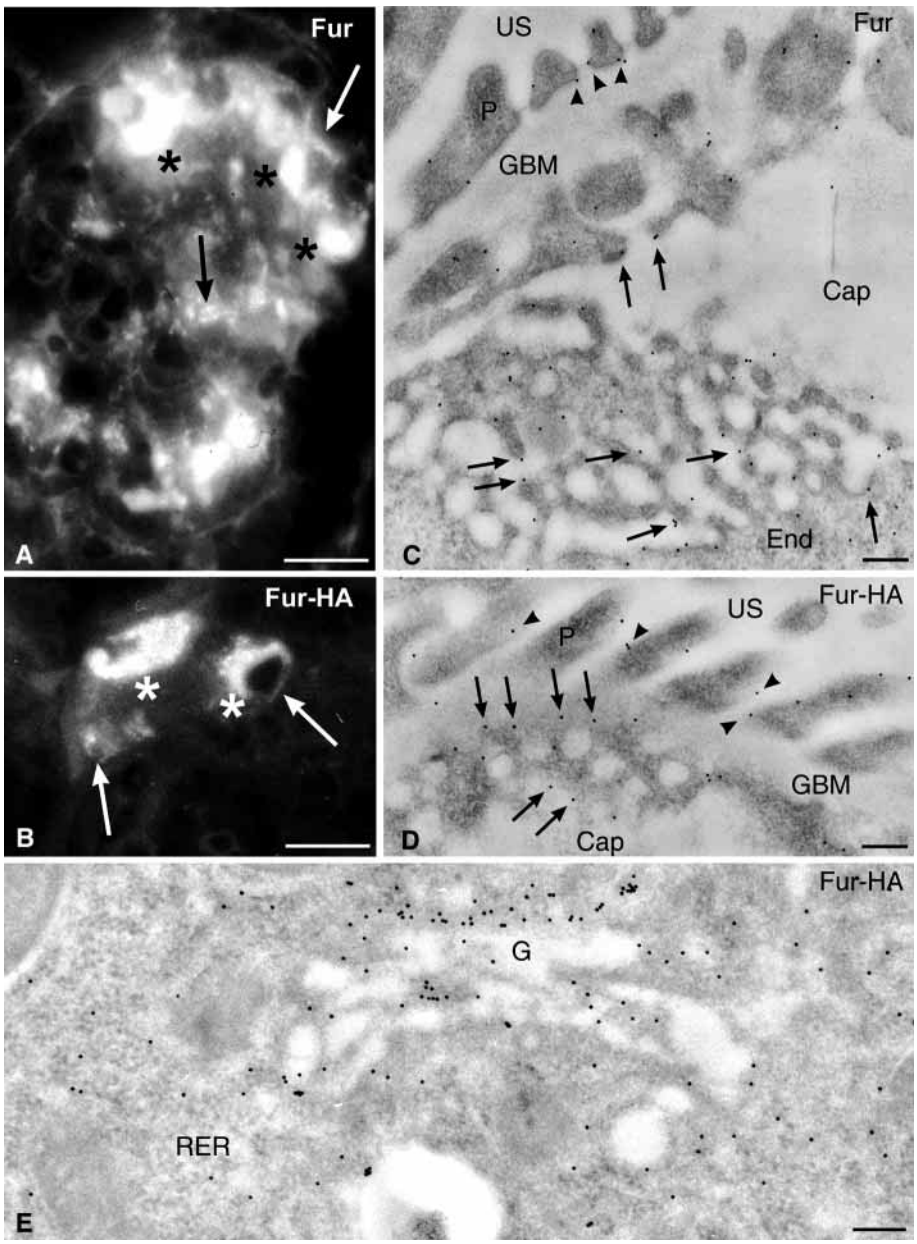

Fig. 2. In vivo overexpressed furin is localized to the Golgi and the cell surface. 24 hours after the injection of plasmids pCDNA3/RSV/Furin (Fur) (A,C) and pCDNA3/RSV/Furin-HA (Fur-HA) (B,D,E) into the tail vein of mice, kidneys were prepared for immunocytochemistry. (A) Furin immunofluorescence on cryosection is found over most of the glomerular cells. (B) Cryosection of kidney transduced with the HA-tagged furin and labeled with the anti-HA antibody; the immunofluorescence is restricted to those cells expressing the exogenous furin-HA. (A,B) The labeling is found in the perinuclear region (asterisks), in vesicular structures and on plasma membranes (arrows). (C-E) Immunogold on furin- and furin-HA-transduced cells. The many gold particles revealing furin (C) and furin-HA (D) are located at the cell surface of podocytes (P) and endothelial cells (End). Labeling is preferentially associated with the slit diaphragms (arrowheads) and endothelial fenestrations (arrows). (E) Golgi apparatus within the cell body of a podocyte transduced with furin-HA. The gold particles are found concentrated on one side of the Golgi, most probably the TGN. (A,B) Bars, 5 μm. (C-E) Bars, 200 nm.

For pCDNA3/RSV/Furin-transduced kidneys, furin was located at the cell surface of glomerular epithelial and endothelial cells (Fig. 2C). Moreover, as reported above, the signal was very frequently associated with endothelial fenestrations and podocyte slit diaphragms (Fig. 2C). Furin was also strongly expressed in the Golgi apparatus (not shown). Furin-HA immunolabeling distinguished the staining caused by endogenous furin. Furin-HA was found at the same locations as the untagged furin. A strong signal was located at the Golgi apparatus, particularly in what appears to be the trans side. Although of lower intensity, the labeling was over endothelial fenestrations and over podocyte slit diaphragms (Fig. 2D,E). Furthermore, the apical domain of foot processes and ablumenal membranes in contact with the GBM were also labeled. Nuclei, mitochondria, basement membranes and the urinary space were almost devoid of gold particles.

Cell surface furin colocalizes and associates with proMT1-MMP

The furin proprotein convertase is thus present in substantial amounts at the surface of cells responsible for glomerular ultrafiltration. These cells synthesize the proteins forming the glomerular basement membrane as well as those regulating the state of this basement membrane. Precursor forms of many regulatory proteins are potential substrates of furin. Among these, MT1-MMP plays a major role in basement membranes turnover. Because furin could intervene in the activation of proMT1-MMP at cell surfaces, we revealed the full-length proMT1-MMP in rat renal glomeruli using an antibody specific to the pro-domain of the enzyme. At the EM level, proMT1-MMP antigenic sites were detected at the surface of glomerular mesangial (not shown), epithelial and endothelial cells (Fig. 3). In podocytes, the labeling was predominantly associated with the lateral membrane domain of foot processes at the level of the slit diaphragms (Fig. 3). In endothelial cells, the labeling was mostly confined to the ablumenal side (Fig. 3, Fig. 4B-D). The gold particles revealing the metalloproteinase antigenic sites were also present in intracellular compartments including the rough endoplasmic reticulum, the Golgi apparatus (Fig. 4A) and podocytic vacuoles (Fig. 4B). ProMT1-MMP immunoreactivity was also found over the GBM, which might reflect the existence of the soluble, shed form of the proenzyme (Kazes et al., 1998). Biochemical results after SDS-PAGE of

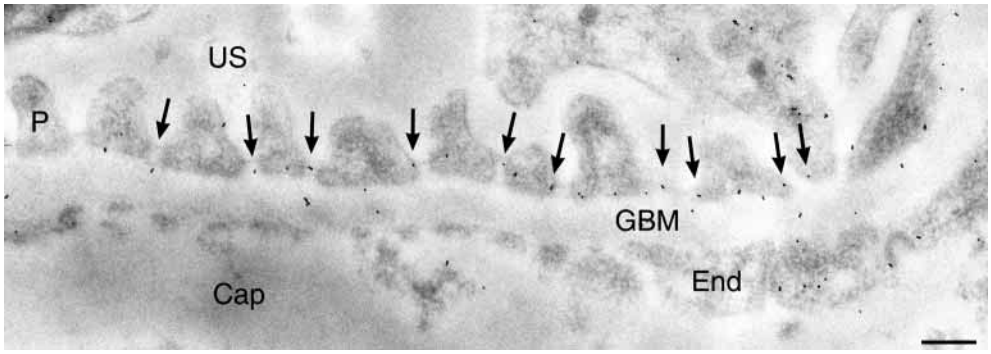

Fig. 3. Subcellular localization of proMT1-MMP to the slit diaphragm. Immunogold on normal rat kidney using the pro-domain-specific anti-proMT1-MMP IgGs with protein-A/10-nm-gold. ProMT1-MMP antigenic sites are mainly located at the base of the podocyte foot processes (P), along the GBM at points of insertion of the slit diaphragms (arrows). The gold particles revealing the precursor are also found over the ablumenal side of the endothelium (End), facing the GBM (not depicted on this micrograph). Abbreviations: Cap, capillary lumen; GBM, glomerular basement membrane; US, urinary space. Bar, 200 nm.

solubilized isolated glomeruli and western blotting using the proMT1-MMP specific antibody, revealed a single band displaying a molecular mass of ~63 kDa. This is in agreement with the molecular mass of the protein in its pro-form and also supports the high specificity of the antibody (Fig. 1F) (Sato et al., 1994).

The detection of proMT1-MMP in the Golgi and at the cell surface in glomerular cells prompted us to investigate its colocalization with furin. When double immunogold labelings were performed on thin sections of rat kidney, 5 nm and 10 nm gold particles (revealing proMT1-MMP and furin, respectively) were often found within 15-25 nm distance suggesting close association of the two antigenic sites. This is illustrated for the Golgi apparatus (Fig. 4A) and intracellular vacuoles of podocytes (Fig. 4B). Moreover, the furin/proMT1-MMP complexes were also present at the slit diaphragm domain (Fig. 4B,C) at the ablumenal side of endothelial cells facing the GBM and in the vicinity of endothelial fenestrations (Fig. 4D). Not all gold particles revealing these proteins were associated; a significant number remained free. Morphometric analysis indicated that ~40% of furin immunogold particles are associated with those for proMT1-MMP at the surface of glomerular cells facing the GBM.

Coimmunoprecipitation experiments were carried out to confirm the association between these enzymes. To overcome possible unstable association, a total glomerular membrane fraction was prepared and membrane proteins were chemically crosslinked with the thiol-cleavable homo-bifunctional reagent DSP. The crosslinked glomerular membranes were subjected to immunoprecipitation with the specific proMT1-MMP antibody and the immunoprecipitated material was analysed by SDS-PAGE, under reducing conditions, and western blotting with anti-furin antibodies. The experiment revealed a 98 kDa protein that coimmunoprecipitated with proMT1-MMP (Fig. 4E, lane 1). Furin was not, however, coimmunoprecipitated with proMT1-MMP in glomerular membranes not treated with DSP (Fig. 4E, lane 2) indicating that their normal association might be disrupted by the isolation and homogenization procedures or that furin has a low binding affinity towards MT1-MMP in the presence of detergents. Upon stripping the nitrocellulose membrane from the antibodies and reprobing it with the anti-proMT1-MMP antibodies (Fig. 4F), one band at 63 kDa was detected independently of chemical crosslinking (Fig. 4F, lanes 1,2). This supports the specificity of the coimmunoprecipitation experiment. Moreover, no protein was revealed using normal rabbit immunoglobulins (Fig. 4E,F, lane 3).

Additional results showing that furin could interact functionally with the proMT1-MMP in kidney glomeruli were obtained from the glomerular membrane digestion study using recombinant furin. Membranes were incubated with soluble furin and analyzed by western blotting to detect the proMT1-MMP. The signal revealing the proenzyme disappeared in the digested membranes indicating that the pro-domain is cleaved by the exogenous soluble furin (Fig. 5A). After stripping, the nitrocellulose membrane was reprobed with the anti-integrin-β3 antibody and the resulting positive signal thus confirmed the integrity of the other membrane proteins (Fig. 5B).

Colocalization and interaction of furin with the αV integrin subunit

Furin has been involved in the endoproteolytic cleavage of the αV integrin subunit precursor (Lehmann et al., 1996; Lissitzky et al., 2000). This post-translational modification converts the αV subunit into its mature form capable of signal transduction (Berthet et al., 2000). Furthermore, furin has an integrin-binding motif (^{391}RGD) and could therefore bind to these ECM receptors at the cell surface as well as in the extracellular milieu. However, this has never been demonstrated. The vitronectin receptor αVβ3 is involved in the activation of MMP2, colocalizes with MT1-MMP and allows the interaction of both metalloproteinases (Puyraimond et al., 2001; Deryugina et al., 2001). The subcellular localization of the αV integrin subunit in the renal glomerulus has been demonstrated previously (Yoon et al., 2001). Our results confirm its presence at the surface of podocytes, particularly on their basal side, around slit diaphragms as well as at the endothelial cell surface (Fig. 6A). Because αV and furin share same locations, we assessed their colocalization. By double immunogold labeling, αV (5 nm gold particles) and furin (10 nm gold particles) were found associated at the podocyte/basement-membrane interface, particularly near the filtration slit diaphragms (Fig. 6B). The same association was also encountered at the surface of endothelial cells but to a lesser extent. Morphometrical analysis indicates that ~30% of cell surface furin is associated with the αV-integrin subunit. No labeling was detected over mitochondria and nuclei and control experiments with

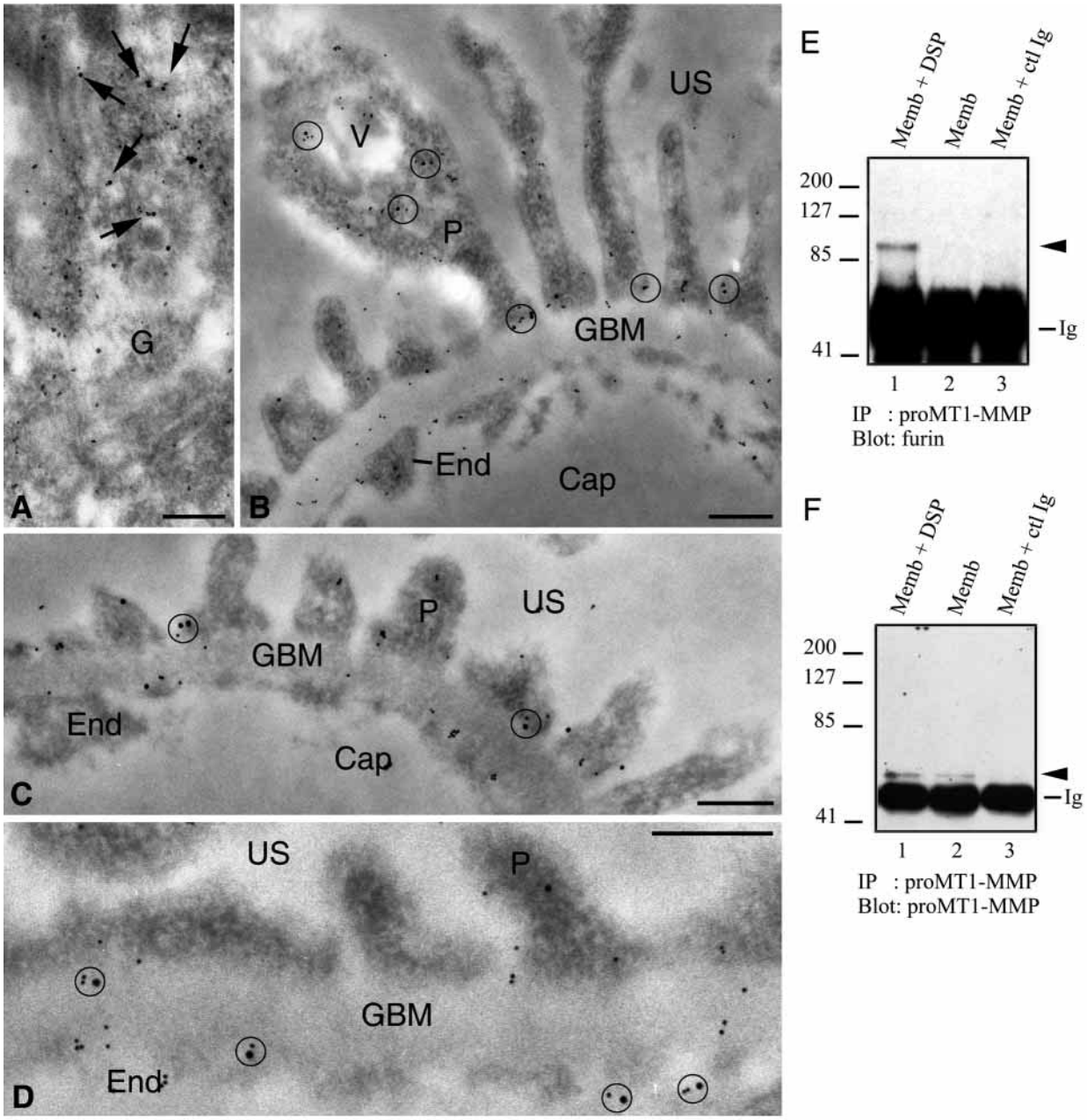

Fig. 4. Furin colocalizes with proMT1-MMP in intracellular compartments as well as at the plasma membrane of glomerular cells. (A-D) Double labeling of furin (10 nm gold particles) and proMT1-MMP (5 nm gold particles). (A) Both labels are present over the Golgi apparatus (G). Several close colocalizations of the large and small gold particles are visible (arrows). (B) Furin and proMT1-MMP labelings are found in close proximity (encircled) over podocytic vacuoles (V) and the basal surface of podocytes. (C) Higher magnification depicting colocalization of furin and proMT1-MMP (encircled) over the slit diaphragm area and the endothelial fenestrations (D). Bars, 200 nm. (E,F) Immunoprecipitation of total membrane fractions of glomeruli (Memb), treated with DSP (lane 1) or untreated (lane 2), with the anti-proMT1-MMP. The material recovered was separated by 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose. (E) Immunoblotting with the rabbit anti-furin-N-terminus antibody revealed one band of ~98 kDa (arrowhead; lane 1). (F) The same blot shown in E, stripped and reprobed for proMT1-MMP. One band at ~63 kDa (arrowhead) is detected independently of chemical crosslinking (lanes 1,2). Notice that, because the immunoprecipitation and the western blotting were conducted with rabbit polyclonal antibodies, there is an immunoglobulin heavy chain band at ~55 kDa in each lane. Lane 3, control. Untreated isolated membrane immunoprecipitated with normal rabbit Ig (ctl Ig).

adsorbed antibodies as well as omitting the primary antibody yield negligible labeling.

Coimmunoprecipitation experiments were carried out to confirm the morphological results. DSP-treated membranes were subjected to coimmunoprecipitation with the specific anti-αV-C-terminus antibody. A 98 kDa protein, corresponding to the molecular mass of furin, coimmunoprecipitated with the αV-antigen-antibody complex (Fig. 6C, lane 1). However, furin

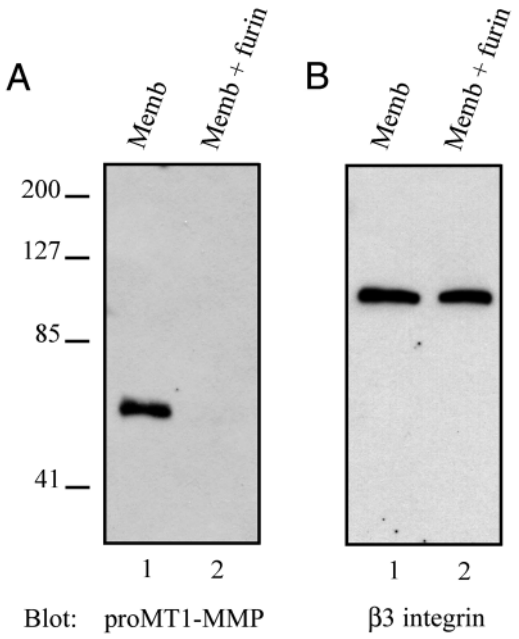

Fig. 5. Cleavage of MT1-MMP pro-domain by furin in isolated glomerular membranes. (A,B) Total membrane fractions of isolated rat glomeruli, untreated (lane 1) or digested with recombinant soluble furin (lane 2), were resolved by 7.5% SDS-PAGE and analysed by western blotting. (A) The 63 kDa band revealing proMT1-MMP in native membranes (lane 1) disappears upon addition of soluble recombinant furin (lane 2), indicating that cleavage of the pro-domain has occurred. (B) In order to assess the integrity of other glomerular membrane proteins, the same blot as in A was stripped and reprobed for the β3 integrin subunit. The appearance of a ~110 kDa band in both lanes indicates the specificity of the limited proteolysis of proMT1-MMP by furin.

was not coimmunoprecipitated with αV in membranes not treated with DSP (Fig. 6C, lane 2), indicating that the furin-αV complex displays weak affinity or that the complex might have been disrupted during the homogenization procedures. When the nitrocellulose membrane was stripped and reprobed with the anti-αV antibody, one band with a molecular mass of ~25 kDa (the molecular mass of the C-terminus of the reduced αV subunit) was detected in the membrane fractions, independently of the chemical crosslinking (Fig. 6D, lane 1,2). This supports the specificity of the coimmunoprecipitation experiment. No protein was detected when immunoprecipitation was carried out with normal rabbit immunoglobulins (Fig. 6C,D, lane 3).

Discussion

Furin is present in two cellular pools, in the Golgi and at the cell surface

Mutagenesis and overexpression studies in cells in culture have unraveled part of the complex trafficking pathway of furin. These studies have established that the furin proprotein convertase is localized mainly in the TGN. They also showed that a very dynamic process takes place because a significant proportion of furin translocates between the Golgi, endosomes and the plasma membrane (Bosshart et al., 1994; Molloy et al., 1999). Thus, one major concern would be to identify the sites where endoproteolytic cleavage of its substrates occurs. The presence of furin at the cell surface was previously deduced from the assessment of the internalization of anti-furin antibodies and the processing of toxins such as the anthrax protective antigen and the pro-aerolysin, for which furin cleavage is a prerequisite for entry into mammalian cells (Klimpel et al., 1992; Molloy et al., 1994; Abrami et al., 1998). In fact, furin can cleave many precursors of toxins and viral glycoproteins at the cell surface and in endosomes, and thus appears to be their Trojan horse (Garten et al., 1994; Molloy et al., 1999). Such a detrimental function is likely to have its physiological counterpart. However, direct proof demonstrating that endogenous furin in normal cells interacts with proproteins at the plasma membrane in a physiological context is still missing.

We have demonstrated that furin is expressed by the rat kidney, particularly by glomerular cells. At the EM level, we identified the precise locations of furin within the Golgi apparatus and at the plasma membrane. Additional evidence showing that furin is indeed gathered at the cell surface was provided by overexpressing the enzyme in glomerular cells using in vivo gene delivery. These results suggest that endogenous and overexpressed furin in intact organ cells are present in two cellular pools, concentrated in the Golgi and present in detectable amounts at the cell surface.

Furin has a short cytosolic domain containing well-defined sequence motifs that direct its sorting to the TGN-endosomal system. The routing of furin depends on its state of phosphorylation and on the interactions of its trafficking motifs with the cellular sorting machinery (Molloy et al., 1999). Moreover, furin delivered to the plasma membrane can interact with the underlying actin cytoskeleton by getting associated to the actin binding protein ABP-280. This scaffolding protein, which organizes actin microfilaments and serves as a docking site for various transmembrane cell surface proteins and intracellular signal transduction proteins, tethers furin to the cell surface and modulates its rate of internalization (Liu et al., 1997). Interestingly, it has been proposed that anchoring furin at the plasma membrane might provide a mechanism for concentrating the protease in discrete regions where efficient extracellular processing could occur (Liu et al., 1997). We did find evidence for such concentrations. Podocyte foot processes are rich in F-actin and other cytoskeletal components such as myosin, α-actinin, vinculin and talin. These cytoskeletal proteins are concentrated along the basal domain of podocytes abutting the GBM and also surround the endothelial fenestrations (Drenckhahn and Franke, 1988). In light of our results on the localization of furin in the glomerulus, it is tempting to speculate that ABP-280 crosslinks furin to F-actin at the podocyte slit diaphragms and at the endothelial fenestrations allowing the protease to act preferentially at these plasmalemmal domains. It is important to realize that MT1-MMP and integrin αVβ3 also interact with F-actin (Galvez et al., 2001; Tsuruta et al., 2002).

Involvement of furin in the activation and trafficking of proMT1-MMP and integrin αV

Conflicting results have been reported about the activation of proMT1-MMP. Cao et al. have shown that the full-length proMT1-MMP could act as an active enzyme, whereas others

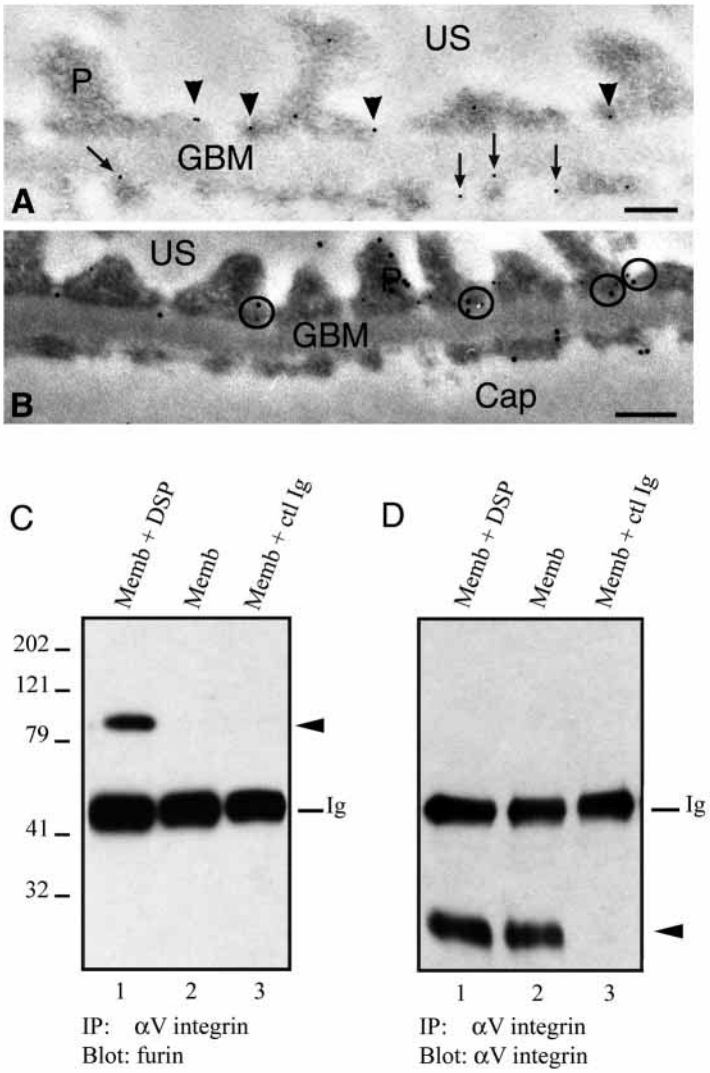

Fig. 6. αV and furin colocalize to the slit diaphragm. (A) Immunogold with the rabbit anti-integrin-αV antibody and protein-A/10-nm-gold. The gold particles are located over the slit diaphragms (arrowheads) and endothelial fenestrations (arrows). (B) Double immunogold labeling. The αV (5 nm gold particles) and furin (10 nm gold particles) colocalize to the base of podocytes and over the slit diaphragms (encircled). Bars, 200 nm. (C,D) Total membrane fractions of isolated glomeruli (Memb), treated with DSP (lane 1) or untreated (lane 2), were immunoprecipitated with the anti-αV-integrin antibody. The resulting material was separated by 10% SDS-PAGE under reducing conditions and analysed by western blotting. (C) The detection of anti-furin antibodies revealed one band of ~98 kDa (arrowhead; lane 1). (D) After stripping the blot shown in C and reprobing it for αV, one band at ~25 kDa (arrowhead), the molecular mass of the reduced αV C-terminus, is detected independently of chemical crosslinking (lanes 1,2). This result supports the specificity of the immunoprecipitations. Lane 3, control. Untreated isolated membrane immunoprecipitated with normal rabbit Ig (ctl Ig).

have shown that proMT1-MMP has to be processed to activate MMP2 (Pei and Weiss, 1996; Yu et al., 1997; Cao et al., 1998; Yana and Weiss, 2000). Recently, several laboratories have generated a wealth of data showing strong correlations between furin activity and those of MT1-MMP and MMP2 (Maquoi et al., 1998; Bassi et al., 2001; de Kleijn et al., 2001). Moreover, several groups have found proMT1-MMP at the plasma membrane of different cell lines, adding weight to the proposition that the pro-domain of MT1-MMP is required for the efficient trafficking of the enzyme to the cell surface (Sato et al., 1994; Sternlicht and Werb, 2001). Still, the identity of the activating enzyme and the exact site of MT1-MMP activation remain obscure. Our EM data demonstrate that proMT1-MMP is indeed localized to the cell surface and our double immunogold labelings and coimmunoprecipitation results indicate that furin associates with proMT1-MMP on plasma membranes of glomerular cells. Moreover, the pro-domain of MT1-MMP in glomerular membrane fractions was cleaved by a soluble form of furin under cell-free conditions, indicating a functional interaction between both enzymes. These results could reconcile the facts that the pro-domain is necessary for MT1-MMP trafficking to the cell surface but that it might have to be cleaved to allow activation. Hence, furin and proMT1-MMP associate at defined plasmalemmal domains of the glomerular cells and could react together to promote GBM turnover either directly through the activation of MT1-MMP or indirectly through the downstream activation of proMMP2. However, our results also show that furin and proMT1-MMP colocalize in the Golgi apparatus and in intracellular post-Golgi/endosomal structures such as podocytic vacuoles. Thus, in addition to the cleavage of MT1-MMP pro-domain, furin could be involved in the trafficking of proMT1-MMP in the secretory pathway. It was recently shown that furin colocalizes with proMT3-MMP and proadamalysin 19 in MDCK cells independently of their enzyme-substrate relationship (Kang et al., 2002a; Kang et al., 2002b). These results imply that pro-domain processing is not an obligatory step in a furin-substrate complex and that furin could escort proproteins to the cell surface.

In addition, we present evidence that furin interacts with the αV integrin subunit. Like other integrin α chains, the αV has a tetrabasic furin recognition site. Furin also holds a cell-adhesion/integrin-binding motif (RGD) in its lumenal domain. Until now, furin has not been found to bind any integrin by virtue of this motif. We here report the association of furin with the αV integrin subunit but cannot attest to whether this is happening through the RGD motif. However, our data also suggest that these proteins interact with low affinity. We can only speculate about the fact that furin could escort MT1-MMP and the αV integrin subunit to the cell surface by binding their pro-domain or other domains with low affinity. It is worth emphasizing that another TGN/cell-surface recycling protein, TGN38, has also been shown to be involved in such trafficking of proteins (the β1 integrin subunit) (Wang and Howell, 2000).

Involvement of furin in GBM turnover

Cell surface binding of furin with proMT1-MMP and integrin αV strongly indicates that it could be involved in an activation cascade leading to GBM degradation. The activation of the soluble proMMP2 occurs on the plasma membrane through a unique multistep pathway involving MT1-MMP, tissue inhibitor of metalloproteinase TIMP-2 and integrin αVβ3

(Deryugina et al., 2001). It appears that MT1-MMP/TIMP-2 complexes and αVβ3 bind proMMP2 and focalize its activation by other unbound, active, MT1-MMP and MMP2 molecules. Furthermore, these docking systems restrict matrix proteolysis to a limited microenvironment on plasma membranes. Accordingly, our results indicate that the association of furin with proMT1-MMP and integrin αV is focused on discrete microdomains at the surface of glomerular cells. This suggests that a mechanism of focalized pericellular proteolysis could be implicated in GBM turnover. Cytoskeletal interactions might be involved in recruiting these complexes to the fenestrations and slit diaphragm area. Other mechanisms that might be involved in the recruitment of furin/proMT1-MMP and furin/αV complexes to specific plasmalemmal sites include their possible association with lipid rafts. MT1-MMP, MMP2 and αVβ3 have all been shown to colocalize with caveolin-1, a major component of caveolae, at the surface of endothelial cells (Puyraimond et al., 2001). Podocyte slit diaphragms are specialized domains containing lipid rafts and it has been shown that many of its components (such as nephrin, podocin and CD2AP) interact with the raft marker caveolin-1 (Schwarz et al., 2001). Recently, Hofmann and colleagues have shown that overexpression of the integrin αVβ3 in an αVβ3-negative cell line greatly increased the processing and activation of proMT1-MMP and, by the same token, that of proMMP2 (Hofmann et al., 2000). Furthermore, they found that MT1-MMP and αVβ3 interact at the cell membrane. This finding could explain our results on the colocalization of furin with αV. In addition to clustering MMP2 near active MT1-MMP, the integrin could also present furin to the MT1-MMP precursor, thereby facilitating proMT1-MMP activation.

The significance of furin in the maturation of a wide array of proproteins in the secretory pathway has been well demonstrated. However, aside from the activation of opportunistic pathogenic entities, its physiological role at the cell surface has remained elusive. In view of recent results, some of its functions at the cell surface are on the verge of being deciphered. We have demonstrated for the first time the interaction of furin with two of its substrates at the cell surface, under physiological conditions. Our data also provide an explanation of some discrepancies in the mechanisms of proMT1-MMP trafficking and activation. We have also shown that, in renal glomeruli, the furin/MT1-MMP axis does exist and might be part of a focalized activation cascade leading to GBM turnover.

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