Biochimica et Biophysica Acta 1813 (2011) 1504–1510

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Release of podocalyxin into the extracellular space
Role of metalloproteinases

Darío Fernández [b], Susana Larrucea [c], Adam Nowakowski [b], Miguel Pericacho [b], Roberto Parrilla [a,b], Matilde S. Ayuso [a,b,*]

[a] Department of Molecular Medicine, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040-Madrid, Spain
[b] CIBER de Enfermedades Raras (CIBERER), Madrid, Spain
[c] Immunology Laboratory, Hospital de Cruces, Baracaldo, Spain

ARTICLE INFO

Article history:
Received 1 March 2011
Received in revised form 13 April 2011
Accepted 10 May 2011
Available online 15 May 2011

Keywords:
Human podocalyxin
Fate of podocalyxin-GFP
Extracellular podocalyxin
CHO cells
Tera-1 cells

ABSTRACT

Podocalyxin (PODXL) is a type I membrane mucoprotein abundantly presented in the epithelial cells (podocytes) of kidney glomeruli where it plays an important role in maintaining the plasma filtration. PODXL is also expressed in other types of cells but its function is ignored. A recombinant soluble fragment of the PODXL ectodomain modifies the signaling of the membrane bound PODXL. Based on this antecedent, we aimed at investigating whether PODXL could be cleaved and released into the extracellular space as a soluble peptide. In this study, we used a fusion protein of human PODXL and green fluorescent protein expressed in CHO cells (CHO-PODXL-GFP) and a human tumor cell (Tera-1) inherently expressing PODXL. PODXL was detected by wide-field microscopy in the Golgi, the plasma membrane and in a vesicular form preferentially located at the leading edges of the cell and also progressing along the filopodium. We detected PODXL in the insoluble and soluble fractions of the extracellular medium of CHO-PODXL-GFP cells. Stimulation of protein kinase C (PKC) by Phorbol-12-myristate-13-acetate (PMA) enhanced the release of PODXL to the extracellular space whereas this effect was prevented either by inhibitors of PKC or specific inhibitors of matrix metalloproteinases. It is concluded that intact PODXL is released to the extracellular space as a cargo of microvesicles and also as a soluble cleaved fragment of ectodomain.

1. Introduction

Podocalyxin (PODXL), also known as Myb-Ets transformed progenitor, is a type I transmembrane sialoglycoprotein of the CD34 family [1], expressed abundantly in the luminal face of the epithelial cells (podocytes) of kidney glomeruli [2,3]. The strong negative charge of PODXL, due to heavy sulfation and sialylation, has been suggested to play a role in maintaining the structural organization of the kidney glomeruli and normal plasma filtration. Podocalyxin is also expressed in tissues other than kidney, like multipotent hematopoietic progenitor cells, vascular endothelium or heart cells [4–6]. Several types of tumor cells express PODXL at rates that appear to correlate with the degree of malignancy [7–9]. Despite its wide expression, the physiological role of extrarenal PODXL is ignored. The soluble recombinant ectodomain of PODXL (PODXLΔ429) counteracts the PODXL effects on cell adhesion and cell–cell interactions and is also capable of triggering cell responses [10]. Thus, we found of interest to investigate whether a cleaved fragment of PODXL was released into the extracellular space under physiological conditions. Some membrane regulatory proteins are cleaved releasing a protein domain to the extracellular medium. In certain cases, the experimental evidence indicates that the released fragment exhibit autocrine or paracrine properties [11].

The present work reports biochemical and morphological evidence showing that intact PODXL is released from CHO cells stably expressing human PODXL or from human tumor cells (Tera1) to the extracellular medium as a cargo of exocytic vesicles and also as a soluble cleaved fragment of ectodomain.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), staurosporine, endoglycosidase H, neuranimidase, PNGaseF and other general reagents were purchased from Sigma (Sigma Chemicals Co.). The matrix metalloproteinase inhibitor Ro31-9790 was provided by Hofmann-LaRoche, Basel, Switzerland, and the specific MMP-14 inhibitor "GACFSIAHECG" as well as its control peptide was a generous gift from Drs. Suojanen and Pirilä, University Helsinki, Finland.

Murine moAbs against human PODXL were either produced in our laboratory, B34D1.3 [12], or purchased (3D3) from Santa Cruz

* Corresponding author at: Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040-Madrid, Spain. Tel.: +34 91 5349154.
E-mail address: msayuso@cib.csic.es (M.S. Ayuso).

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.05.009

Biotechnology (Santa Cruz, CA). Anti-GFP was also from Santa Cruz Biotechnology and horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse IgG from Bio-Rad Laboratories (Hercules, CA).

2.2. Cell lines and culture conditions

CHO cells were maintained in DMEM medium (Gibco), supplemented with 10% FCS (ICN), hydroxytryptamin (HT), penicillin G 100 U/mL (Gibco) and streptomycin 100 μg/mL (Gibco), at 37 °C and 5% CO_2. Tera-1 cells, originated from a germ cell human tumor of seminomatous origin, were grown in McCoy's 5A medium containing 10% fetal calf serum and 1 mmol/L glutamine.

2.3. Preparation of expression plasmids encoding a fusion protein of human podocalyxin and green fluorescent protein

The construct encoding a fusion protein with GFP linked in-frame at the carboxylic end of PODXL was previously described [10]. To generate a fusion protein in which GFP was linked in frame upstream of PODXL, we first inserted the sequence of the PODXL signal peptide at the 5′ end of GFP in the plasmid pEGFP-N1. For this purpose the 1–178 bp region of PODXL was amplified with the primers:

sp hpodxl SacII Kozak ATG:

5′-CCC CGC GGC GAC GCC ACC ATG GGC TGC GCG-3′

sp hpodxl BamH1 woSC:

5′-CGG CGA GGA TCC CGA CGG CAG CAG CGG CGG-3′

The 66 bp PCR amplification product was ligated into the SacII and BamH I of pCR 2.1-TOPO. Secondly the stop codon of the GFP sequence of pEGFP-N1 was removed by using the site directed mutagenesis kit QuickChangeII (Stratagene, La Jolla, CA) using the following primers:

5′ GCA TGG ACG AGC TGT ACA AGT TAA GCG GCC GCT CGC CGT CGC
3′ GCG ACG GCG AGC GGC CGC TTA ACT TGT ACA GCT CGT CCA TGC

Finally, we amplified by PCR a DNA fragment from the expression plasmid pcDNA3-podocalyxin cDNA using the primers:

5′ pcDNA3 hpodxl 1085–1114 Not I
5′-CTG CGC GGC CGC TCG CCG TCG CCG TCG CCG-3′
3′ pcDNA3 hpodxl 2611–2640 Not I
5′-CTG GAG GCC ACC GGC GCG GCC GCC TAG AGG-3′

The amplified DNA fragment was cloned into the pCR 2.1-TOPO (Invitrogen), digested with NotI and ligated to the pEGFP-N1 vector previously digested with the same restriction enzyme. All the steps were verified by DNA sequencing.

2.4. Transfection of the podocalyxin transgene into Chinese hamster ovary (CHO) cells

CHO cells were stably transfected with PODXL-GFP or GFP-PODXL by the calcium-phosphate procedure using G-418 (400 μg/mL) in the selection medium. The transfected cells were isolated by cell sorting.

2.5. Cell imaging of CHO-PODXL-GFP and CHO-GFP-PODXL by wide-field microscopy

CHO cells stably expressing PODXL-GFP or GFP-PODXL were grown in DMEM medium (Gibco) containing 10% FCS, hydroxytryptamin (HT), penicillin G 100 U/mL (Gibco) and streptomycin 100 μg/mL (Gibco), at 37 °C and 5% CO_2, in 25 mm microscopic quality plates (MatTek, USA). To take images *in vivo* the cells were suspended in serum-free medium, and the plate fitted into a thermostatic controlled heating unit (37 °C) of a Leica AF7000 Fluorescence Advanced Widefield System, with a Live Data Mode interactive data recording allowing job-sequencing and online evaluation. Images were taken with a CCD camera Hamamatsu 9100-02. A Plan-Apochromate 63×/1.4, glycerol immersion lens, or a 100×, oil immersion lens, were used. The pair of wavelengths 470–490 nm (excitation/emission) was used to visualize GFP.

2.6. Western blotting

Cell lysates from CHO-GFP or CHO-PODXL-GFP were prepared in modified RIPA buffer (50 mM pH 7.4 Tris–HCl, 1% NP-40, 0.25% Na-deoxicholate, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3PO_4, 1 mM NaF and a protease inhibitor cocktail (Roche, Indianapolis, IN) and loaded into 7.5% SDS-polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose membranes and visualized by incubation with an anti-PODXL or anti-GFP moAb for 2 h at room temperature, followed by HRP-conjugated secondary goat anti-mouse IgG. Blots were developed using an enhanced chemiluminescence's detection system (ECL).

2.7. Preparation of microvesicles (MV) from extracellular medium and detection of PODXL

For immunoprecipitation of culture medium, the cells were cultured in 6 well plates in DMEM medium with 10% FCS, hydroxytryptamin (HT), and 100 U/mL penicillin/100 μg/mL streptomycin, at 37 °C and 5% CO_2. When 90% confluence was reached, cells were washed three times with PBS and incubated in 3 mL of DMEM serum-free medium for 2 h, collected and centrifuged at 1800×*g* for 20 min at 4 °C and the supernatant at 100,000×*g* for 1 h at 4 °C. The supernatants were either concentrated down to 500 μL using Amicon centriplus YM-3 columns (Amicon, Bedford, Ma) or immunoprecipitated by incubation overnight at 4 °C with 2 μg of purified anti-PODXL (B34.D1.3). After adding 50 μL of protein G-sepharose (Pharmacia) the samples were incubated at 4 °C for 2 h and centrifuged at 1800×*g* for 15 min. The pellet was washed three times with modified RIPA-buffer and suspended in 50 mL of Laemmli buffer 2× [13], boiled for 5 min and centrifuged. The whole supernatant was electrophoresed in SDS-PAGE (7.5%) and blotted as described above. Extracellular microvesicles were obtained by centrifugation of the medium at 100,000×*g* and suspending the pellet directly in 2× Laemmli buffer.

2.8. Analysis of CHO-PODXL-GFP cells and extracellular microvesicles by electron microscopy

The microvesicle-containing extracellular medium was obtained by centrifugation at 100,000×*g* and the pellet was resuspended in 50 μL of 0.4 M HEPES (Gibco), mounted on carbon grids by electric charge (Glow Discharge), washed, stained with 2% uranyl acetate, visualized with a transmission electron microscope Jeol 1230 at 100 kV and images capture with a digital camera.

3. Results

3.1. Morphological features of CHO cells expressing human podocalyxin

We analyzed *in vivo*, by wide field microscopy, the morphological features of cells stably transfected with either GFP (CHO-GFP) or PODXL linked in frame to GFP (CHO-PODXL-GFP). The cells were seeded into glass-bottom culture dishes and allowed to stand in an incubator at 37 °C, under 5% CO_2. The GFP fluorescence was homogeneously diffuse in CHO-GFP cells (Fig. 1, left column) where in the PODXL-GFP the fluorescence was perinuclear, likely in the Golgi apparatus, as expected of a glycosylated protein like PODXL, in the plasma membrane and in a cytoplasmic adopting a vesicular form (Fig. 2, right column). A distinct feature of the PODXL-GFP containing vesicles was their location at the leading edge of the cell, the sides of intercellular contact (Fig. 2) and also progressing along the filopodia,

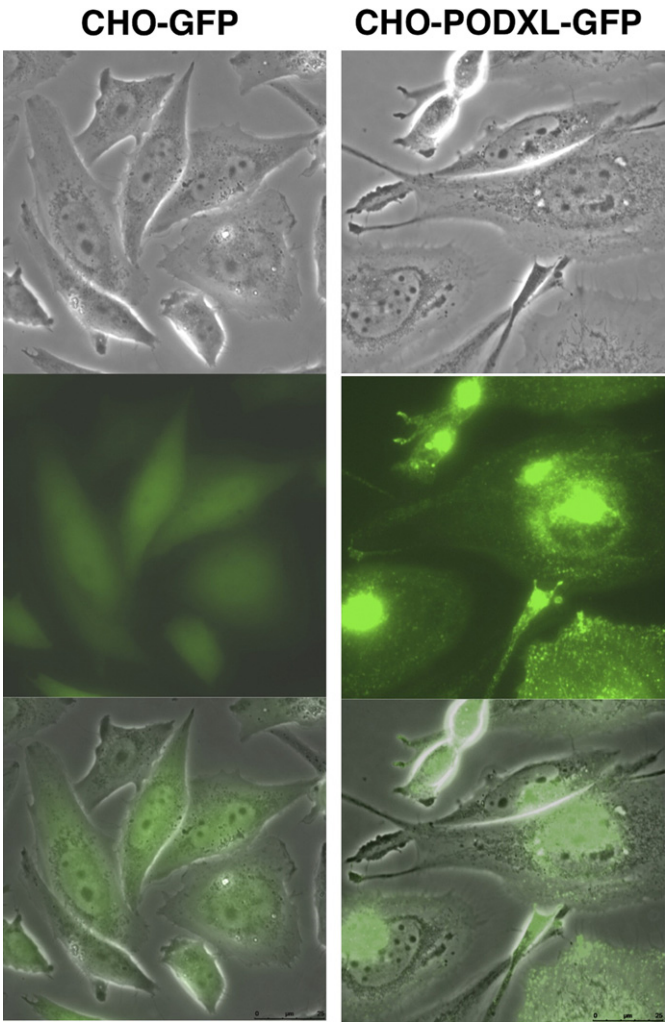

Fig. 1. Phase and fluorescence images of live CHO-GFP and live CHO-PODXL-GFP cells. Cells were seeded in glass-bottom culture dishes coated with fibronectin and allowed to stand in an incubator at 37 °C, under 5% CO_2 as described in Materials and methods. Phase contrast and fluorescence images of CHO-GFP (left column) and CHO-PODXL-GFP cells (right column), ~8 h after seeding. In CHO-GFP the fluorescence was diffuse, absent in the Golgi or in any other distinct structure. In contrast, PODXL-GFP fluorescence was clearly detected in the Golgi apparatus, plasma membrane and cytoplasmic vesicles. Images were captured using a Leica wide field setup with a ×100 oil immersion objective.

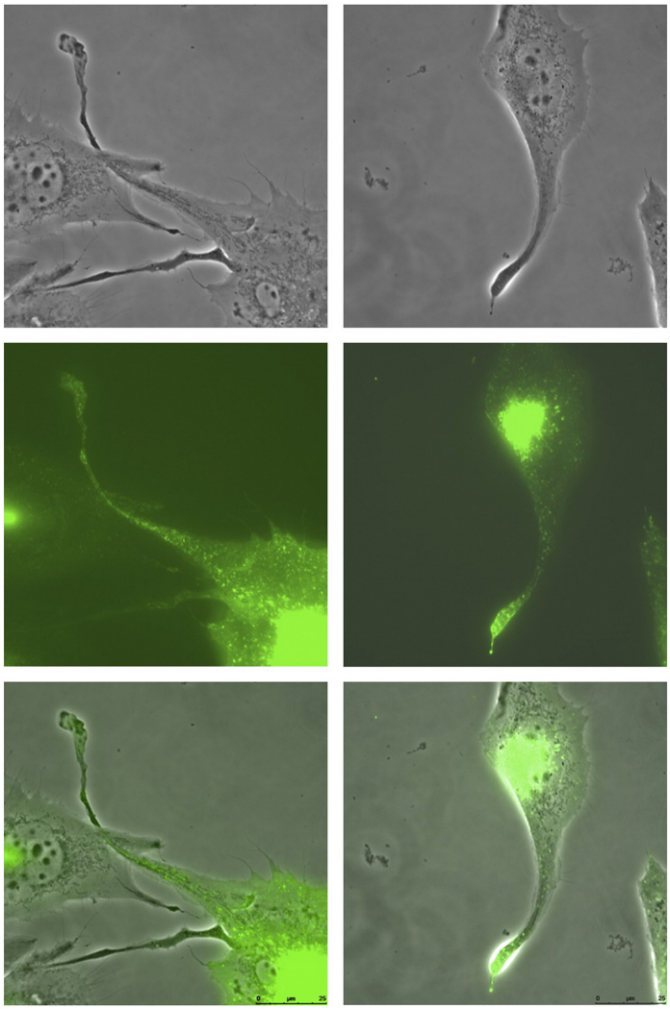

Fig. 2. Images of live CHO-PODXL-GFP. Phase and fluorescence images of PODXL-GFP cells. Experimental conditions were those described in Fig. 1. Fluorescence of PODXL-GFP was located at the Golgi apparatus, plasma membrane and in cytoplasmic vesicles preferentially located at the leading edges of cells and along the filopodium. Images taken with a Leica wide field setup using a ×100 oil immersion objective.

accumulating at the tip to finally be released into the extracellular space (Figs. 2,3). These experiments suggest that PODXL could be released into the extracellular medium as a cargo of microvesicles.

3.2. Immunodetection of podocalyxin in total cell lysates and extracellular medium of CHO-PODXL-GFP cells

Blotting proteins from PODXL-GFP cell lysates with the anti-PODXL, B34D1.3, moAb revealed the presence of two distinct bands of ~160 and ~200 kDa (Fig. 4A). In contrast only the band of ~200 kDa was detected, when blotting with anti-GFP (Fig. 4B).

To identify the nature of the anti-PODXL reacting bands, cell lysates were immunoprecipitated with the anti-PODXL moAb 3D3 and electrophoresed in SDS-PAGE. Three bands were detected in the anti-PODXL immunoprecipitate as compared with the immunoprecipitate made with an irrelevant IgG (Fig. S1). The three bands were excised from the gels and identified by mass spectrometry (Table S1). Band 1 was identified as the fusion protein PODXL-GFP, band 2 as PODXL and the third one was identified as the constant region of the IgG1 heavy chain.

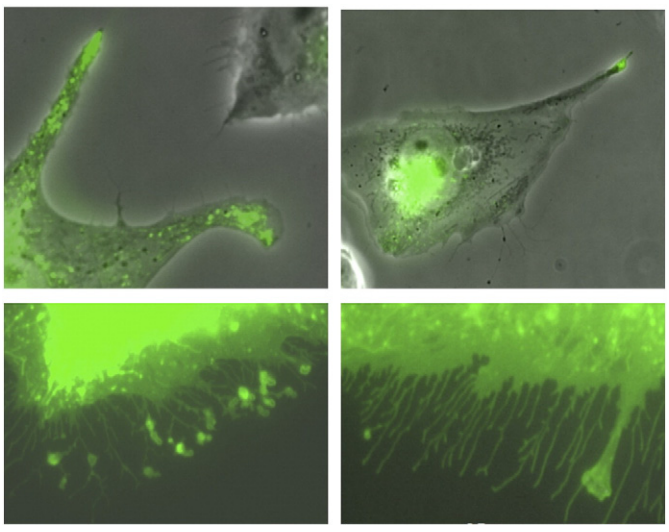

Fig. 3. Images of live CHO-PODXL-GFP. Experimental conditions were those described in Fig. 1. These images illustrate the progression of PODXL-fluorescent vesicles along the filopodia to apparently be released into the extracellular space. Images were taken with a Leica wide-field setup using a ×100 immersion oil objective.

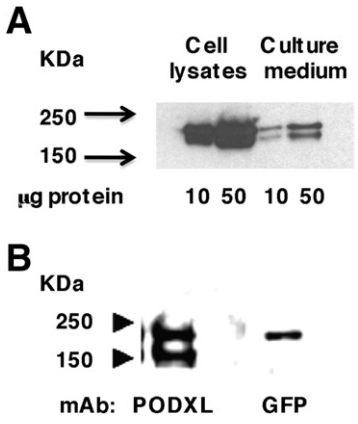

Fig. 4. Detection of extracellular PODXL. CHO-PODXL-GFP cells were incubated as described in Materials and methods. To determine whether PODXL was released into the extracellular space, the cells were cultured for 18 h in 6 well plates in 7 mL of serum-free culture medium. At the end of the incubation the cells were harvested in 500 µL of lysis buffer and the culture medium was concentrated by centrifugation. A) The indicated amounts of protein of both, lysates and concentrated extracellular medium were electrophoresed in SDS-PAGE and blotted with anti-PODXL moAb B34D1.3. B) Proteins from cell lysates blotted with either anti-PODXL or anti-GFP.

PODXL-immunoreactive material was also found in the extracellular space. To investigate the mechanism of PODXL release to the medium, we performed experiments using CHO cells expressing podocalyxin with GFP fused in frame at either the carboxy (CHO-PODXL-GFP) or the amino-terminus (CHO-GFP-PODXL). We have verified the presence of both types of fusion proteins in the plasma membrane by either flow cytometry or by wide field microscopy (results not shown). Digestions with endoglycosidase H, neuraminidase and PGCaseF indicated that both types of fusion proteins exhibited identical patterns of glycosylation (Fig. S2). Regardless of whether protein samples from CHO-PODXL-GFP or CHO-GFP-PODXL lysates were blotted with anti-PODXL or with anti-GFP, in both cases PODXL was detected as a band of similar size than the native PODXL from the human teratocarcinoma Tera-1 cells [14] (Fig. 5).

PODXL was also found in the extracellular medium of both, CHO-PODXL-GFP or CHO-GFP-PODXL when blotting with anti-PODXL, indicating that PODXL was released from the cell as a full size molecule (Fig. 6). However, PODXL was undetected in the extracellular medium of CHO-PODXL-GFP cells when the proteins were blotted with anti-GFP (Fig. 6). This observation suggests a deletion of the carboxy terminus of PODXL-GFP. As indicated above, intact PODXL, likely forming multimeric complexes, could have been exported as a cargo of microvesicles.

In fact, we detected PODXL by western blot of microvesicles (~100 µm) purified from the insoluble extracellular fraction of CHO-PODXL-GFP cells (Fig. 7A) or TERA-1 cells (Fig. 7B). Moreover, we observed the release of microvesicles content through the plasma membrane of CHO-PODXL-GFP by electron microcopy (Fig. 7C). Panels 7C(c-f), show different steps in the release of microvesicles content.

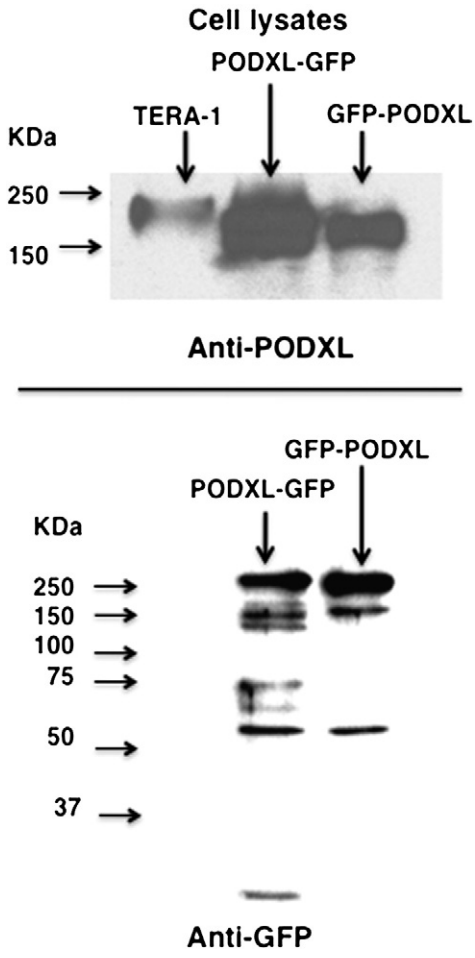

Fig. 5. Detection of PODXL in cell lysates of Tera-1, CHO-PODXL-GFP and CHO-GFP-PODXL cells. Tera-1 and CHO cells producing human PODXL-GFP linked to either the amino or the carboxylic ends were cultured and lysates prepared as described in Materials and methods. 50 µg of lysate protein was electrophoresed in SDS-PAGE and blotted with anti-PODXL moAb B34D1.3 (upper panel) or with anti-GFP (lower panel) as indicated.

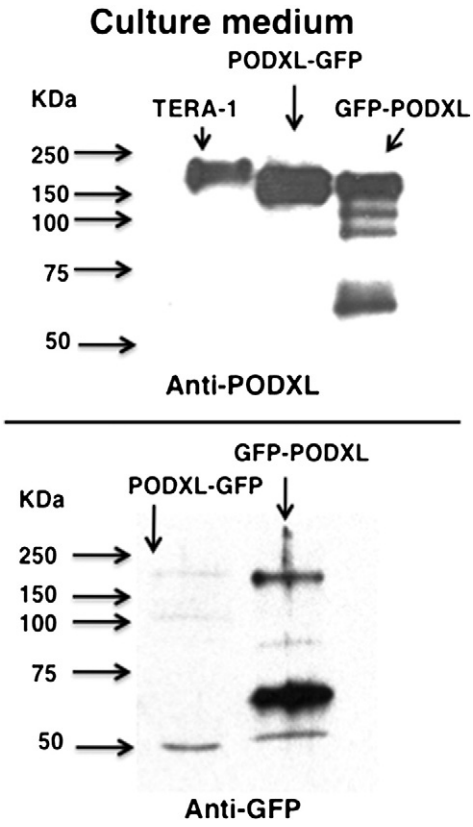

Fig. 6. Detection of PODXL in the extracellular medium of cultured CHO-PODXL-GFP, CHO-GFP-PODXL or Tera-1 cells. Culture medium from Tera-1 and CHO cells stably producing human PODXL-GFP fused to either the amino or the carboxylic ends were concentrated by centrifugation and processed as described in Materials and methods. 50 µL of concentrated extracellular medium were electrophoresed in SDS-PAGE and blotted with anti-PODXL moAb B34D1.3 (upper panel) or with anti-GFP (lower panel).

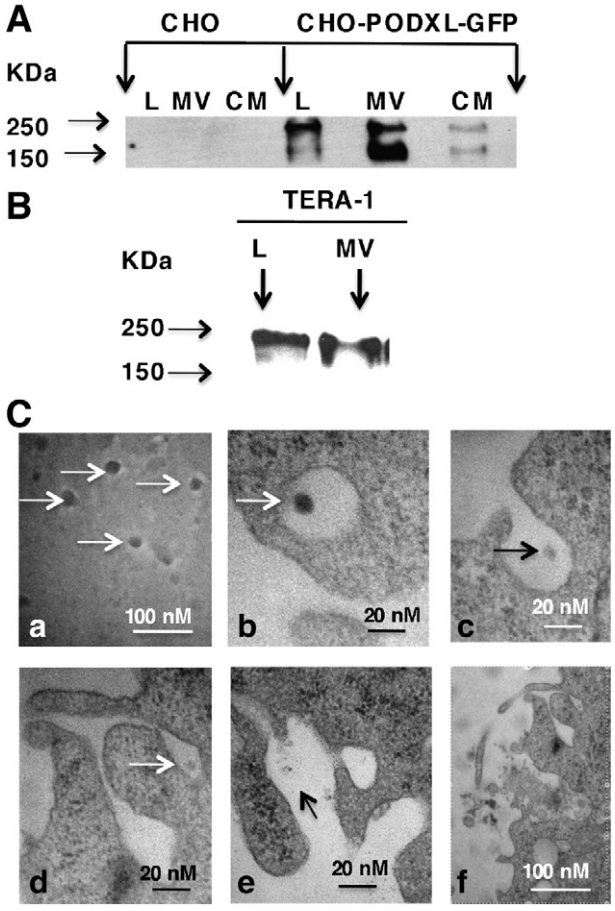

Fig. 7. Identification of PODXL in both the insoluble (microvesicles) and soluble supernatant fractions of culture medium from CHO-GFP, CHO-PODXL-GFP or Tera-1 cells. The experimental conditions were those described in Fig. 5. Culture medium of either CHO-GFP (A), CHO-PODXL-GFP (A) or Tera 1 cells (B) (7 mL) were centrifuged at 1800×*g* for 15 min and the supernatant at 100,000×*g* for 30 min. The pellet (MV) was resuspended in 500 mL of lysis buffer and the supernatant (CM) concentrated to 500 μL. 50 μg of protein from cell lysates, MV and CM were electrophoresed in SDS-PAGE and blotted with anti-PODXL moAb B34D1.3. (C) Electron micrographs of CHO-PODXL cells. Panels c-f show different steps in the release of microvesicles content. Magnifications: (a,f) 100,000; (b–e) 300,000.

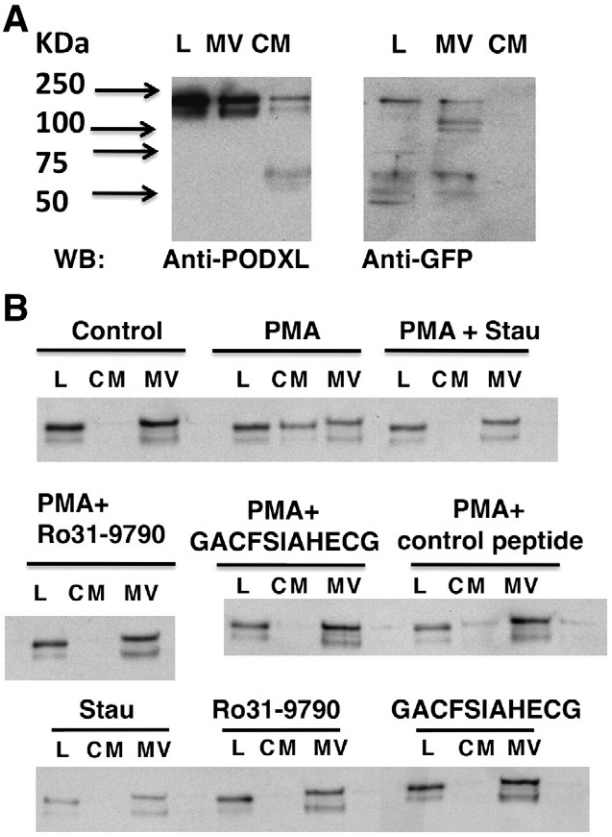

Fig. 8. Detection of PODXL or GFP-containing PODXL fragments in cell lysates, extracellular microvesicles (MV) and in the soluble supernatant fraction of culture medium (CM) of CHO-PODXL-GFP cells. A) Cells were cultured in 30 mm plates with 3 mL of serum-free medium, for 2 h. The cells were harvested and suspended in 50 μL of lysis buffer. The culture medium was processed as described in Fig. 5 and the microvesicles (MV) suspended in 50 μL of lysis buffer. The culture medium (CM) was immunoprecipitated with 2 μg of purified anti-PODXL moAb (B34D1.3) as described in Materials and methods. 50 μg of lysate proteins, 50 μL of MV and 50 μL of CM immunoprecipitated proteins were electrophoresed in SDS-PAGE and blotted with anti-PODXL moAb B34D1.3 or with anti-GFP Ab. B) To determine whether the PKC activity or inhibitors or matrix metalloproteinase altered the excretion of PODXL, CHO-PODXL-GFP cells were incubated for 2 h in serum-free medium in the presence or the absence of 100 nM phorbol-esters (PMA), 100 nM staurosporine, 100 nM of both "GACFSIAHECG" and control peptide, 10 nM Ro31-9790, or the indicated combinations of these reagents. At the end of the incubation the cell lysates (L) were processed as indicated before. The extracellular medium was separated in two fractions: particulate (microvesicles) (MV) and particle-free supernatant (CM). The latter was immunoprecipitated with anti-PODXL moAb and the precipitates electrophoresed and blotted with anti-PODXL.

The finding of similar distribution of PODXL in total cell lysates or in soluble (not shown) or insoluble fractions of culture medium of Tera-1 cells (Fig. 7B) seems to validate our results on cells expressing recombinant human PODXL.

PODXL reactivity from the insoluble fraction (microvesicles) of extracellular medium blotted with anti-GFP showed a similar size than fully glycosilated PODXL (Fig. 8A). On the contrary, the size of the PODXL-immunoreactive protein of the soluble extracellular fraction was smaller than native PODXL, suggesting it was a form lacking the carboxylic-GFP terminus. We estimate that the PODXL content in microvesicles could account for less than <1% of the total cellular content, while the extracellular content would be less than <0.3%.

Fig. S3 depicts the estimated matrix metalloproteinases (MMP) sites in the PODXL molecule, analyzed according to the protease cleavage sites described by Turk et al. 2001 [15]. Three of the PODXL reactive proteins in cell lysates showed apparent molecular weights that seem to agree with the estimated protein subproducts of PODXL digestion by matrix metalloproteinases MMP-1,2,9 and 14 (Fig. S3). MMP-1 is collagenase, MMP-2 and 9 are gelatinases and MMP-14 is a membrane type metalloproteinase (MT-MMP-14) [16] and all of them have been shown to be active in CHO cells [17–19]. The additional bands of ≥80 and 50 KDa detected in concentrates of the particle-free soluble fraction of extracellular medium may be irrelevant since they were also found in cell transfected only with GFP (Fig. S3). To evaluate the importance of metalloproteinases on the fate of cellular PODXL, we studied the effect of activating the protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) with or without inhibitors of PKC. We have also studied the effects of the general inhibitor of zinc–dependent matrix metalloproteinases, Ro31–9790, previously shown to be effective in CHO cells [17], or the specific inhibitor of MMP-14, the peptide "GACFSIAHECGA" [20], on CHO-PODXL-GFP cells incubated for 2 h, in serum-free medium. Activation of protein kinase C increased the amount of PODXL-immunoreactive material in anti-PODXL immunoprecipitates of the soluble, particle-free, supernatants, effect that was reversed by staurosporine or by the inhibitors of metalloproteinases Ro31-9790 or "GACFSIAHECG" (Fig. 8B).

4. Discussion

By wide-field microscopy of fully extended migrating cells, we detected PODXL-GFP-containing microvesicles at the leading edges of

the cell, regions of intercellular contact, and moving forward along the filopodia. The microscopic analysis suggested that PODXL was released to the extracellular space as microvesicles of ≤100 nM.

The finding of full size, glycosylated, PODXL in the extracellular insoluble particulate fraction (microvesicles) indicates that PODXL was released as a cargo of microvesicles, presumably forming macromolecular complexes with other exported proteins.

The analysis of soluble and insoluble extracellular fractions of cells expressing PODXL with GFP linked to either the carboxylic (PODXL-GFP) or the amino-terminal end (GFP-PODXL) and using anti-PODXL or anti-GFP, allowed the detection of full size glycosylated PODXL-GFP as well as a smaller soluble protein reacting with anti-PODXL but not with anti-GFP, indicating it was an ectodomain fragment.

The release of PODXL is in line with observations made in other plasma membrane regulatory proteins that undergo a process of cleavage releasing an ectodomain fragment to the extracellular space [11]. Plasma membrane proteins like CD40L [21], P-selectin [22], growth hormone receptor [17], epidermal growth factor (EGF) [23] or tumor necrosis factor (TNF) families [24], among others, are synthesized as plasma transmembrane forms that undergo a process of cleavage releasing a soluble fragment of the ectodomain. The fragment of PODXL released could modulate the function of ligands acting as either antagonists or agonists in the formation of active signaling complexes, as it has been suggested for other proteins [25].

The soluble fragment of PODXL might have been cleaved by metalloproteinases [26]. Based on the matrix metalloproteinases (MMP) excision sites [15] we identify three potential cleavage sites in the PODXL protein sequence that could eventually be responsible for some of the PODXL peptide subproducts detected in cell lysates. Since the activity of metalloproteinases is stimulated by PKC, we investigated the role played by MMPs in releasing PODXL by stimulating the activity of PKC or using specific inhibitors of matrix metalloproteinases. Activation of PKC by PMA increased the amount of podocalyxin in immunoprecipitates of particle-free supernatant of extracellular medium. On the contrary, inhibition of PKC by staurosporine, matrix metalloproteinase inhibitors, like Ro31-9790, or the peptide inhibitor "GACFSIAHECG", they both prevented the stimulatory effect of PMA. These observations suggest that MMPs-mediated cleavage is a relevant mechanism for the cellular release of soluble PODXL. We estimate that, after 2 h of cell incubation, the PODXL content in the extracellular insoluble material was ≤1% of the total cell PODXL, whereas the soluble PODXL content was ≤0.3%. These values are in line with the 2% reported for other shedded membrane proteins whose released fragments are functionally active [11,18]. The physiopathological significance of our findings is consonant with the finding of a PODXL fragment of 2.3 kDa, comprising part of the extracellular and transmembrane domains, found in the extracellular medium of colon cancer cells [27].

Urinary excretion of PODXL is a useful marker for detection and progression of kidney inflammatory disorders [28]. The need of detergents to solubilize the urinary sediments of patients indicates the association of PODXL with insoluble cellular materials. Recently, Hara et al. reported the presence of intact PODXL in the urinary sediment of nephritic patients as "podocalyxin positive granular structures (PPGS)", thought to originate from tip vesiculation of podocyte microvilli [29]. This observation agrees with the normal location of podocalyxin at the luminal face (microvilli) of glomerular podocytes and with the cellular destruction caused by the inflammation injury. Moreover, Hara et al. failed to find podocalyxin in exosomes, suggesting their findings may not be applicable to normal physiological conditions or they may not reflect the trafficking of podocalyxin in cells other than podocytes.

The soluble ectodomain of PODXL (PODXLΔ_{429}) inhibits the PODXL responses [10]. Thus, it seems plausible to consider PODXL as another example of membrane protein with a duality of functions: as a full size membrane protein sending inward signaling, or as a soluble portion of extracellular domain exerting antagonistic effects and facilitating the intercellular communication. The transfer of signaling molecules from tumor cells microvesicles into other type of cells has been recently demonstrated [30,31]. Thus, the possibility should be considered that the released PODXL into the extracellular space could serve this function.

To conclude, the morphological and biochemical data presented in this work supports the idea that PODXL is secreted into the extracellular medium as a cargo of microvesicles either as an intact protein or as a cleaved fragment of the ectodomain. The presence of both forms of extracellular PODXL should be taken into account when the physiological action of this molecule was to be evaluated.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.05.009.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Innovation (SAF2005-01261 and SAF2007-61701). The CIBER de Enfermedades Raras (CIBERER) is an initiative of the ISCIII.

References

[1] J.S. Nielsen, M.L. Graves, S. Chelliah, A.W. Vogl, C.D. Roskelley, K.M. McNagny, The CD34-related molecule podocalyxin is a potent inducer of microvillus formation, PLoS One 2 (2007) e237.
[2] D. Kerjaschki, D.J. Sharkey, M.G. Farquhar, Identification and characterization of podocalyxin—the major sialoprotein of the renal glomerular epithelial cell, J. Cell Biol. 98 (1984) 1591–1596.
[3] M.W. Seiler, H.G. Rennke, M.A. Venkatachalam, R.S. Cotran, Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium, Lab. Invest. 36 (1977) 48–61.
[4] L. Kerosuo, E. Juvonen, R. Alitalo, M. Gylling, D. Kerjaschki, A. Miettinen, Podocalyxin in human haematopoietic cells, Br. J. Haematol. 124 (2004) 809–818.
[5] D.B. Kershaw, P.E. Thomas, B.L. Wharram, M. Goyal, J.E. Wiggins, C.I. Whiteside, R.C. Wiggins, Molecular cloning, expression, and characterization of podocalyxin-like protein 1 from rabbit as a transmembrane protein of glomerular podocytes and vascular endothelium, J. Biol. Chem. 270 (1995) 29439–29446.
[6] D.B. Kershaw, S.G. Beck, B.L. Wharram, J.E. Wiggins, M. Goyal, P.E. Thomas, R.C. Wiggins, Molecular cloning and characterization of human podocalyxin-like protein. Orthologous relationship to rabbit PCLP1 and rat podocalyxin, J. Biol. Chem. 272 (1997) 15708–15714.
[7] W.M. Schopperle, D.B. Kershaw, W.C. DeWolf, Human embryonal carcinoma tumor antigen, Gp200/GCTM-2, is podocalyxin, Biochem. Biophys. Res. Commun. 300 (2003) 285–290.
[8] G. Casey, P.J. Neville, X. Liu, S.J. Plummer, M.S. Cicek, L.M. Krumroy, A.P. Curran, M.R. McGreevy, W.J. Catalona, E.A. Klein, J.S. Witte, Podocalyxin variants and risk of prostate cancer and tumor aggressiveness, Hum. Mol. Genet. 15 (2006) 735–741.
[9] A. Somasiri, J.S. Nielsen, N. Makretsov, M.L. McCoy, L. Prentice, C.B. Gilks, S.K. Chia, K.A. Gelmon, D.B. Kershaw, D.G. Huntsman, K.M. McNagny, C.D. Roskelley, Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression, Cancer Res. 64 (2004) 5068–5073.
[10] S. Larrucea, N. Butta, E.G. Arias-Salgado, S. Alonso-Martin, M.S. Ayuso, R. Parrilla, Expression of podocalyxin enhances the adherence, migration, and intercellular communication of cells, Exp. Cell Res. 314 (2008) 2004–2015.
[11] J. Arribas, A. Borroto, Protein ectodomain shedding, Chem. Rev. 102 (2002) 4627–4638.
[12] R.B. Rodriguez, N. Butta, S. Larrucea, S. Alonso, R. Parrilla, Production and characterization of murine monoclonal antibodies against human podocalyxin, Tissue Antigens 68 (2006) 407–417.
[13] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[14] D.L. Bronson, E.E. Fraley, J. Fogh, S.S. Kalter, Induction of retrovirus particles in human testicular tumor (Tera-1) cell cultures: an electron microscopic study, J. Natl. Cancer Inst. 63 (1979) 337–339.
[15] B.E. Turk, L.L. Huang, E.T. Piro, L.C. Cantley, Determination of protease cleavage site motifs using mixture-based oriented peptide libraries, Nat. Biotechnol. 19 (2001) 661–667.
[16] M. Egeblad, Z. Werb, New functions for the matrix metalloproteinases in cancer progression, Nat. Rev. Cancer 2 (2002) 161–174.
[17] T. Amit, Z. Hochberg, M. Yogev-Falach, M.B. Youdim, R.J. Barkey, Shedding of growth hormone-binding protein is inhibited by hydroxamic acid-based protease inhibitors: proposed mechanism of activation of growth hormone-binding protein secretase, J. Endocrinol. 169 (2001) 397–407.
[18] J. Arribas, L. Coodly, P. Vollmer, T.K. Kishimoto, S. Rose-John, J. Massague, Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors, J. Biol. Chem. 271 (1996) 11376–11382.
[19] J. Mols, C. Peeters-Joris, R. Wattiez, S.N. Agathos, Y.J. Schneider, Recombinant interferon-gamma secreted by Chinese hamster ovary-320 cells cultivated in suspension in protein-free media is protected against extracellular proteolysis by

the expression of natural protease inhibitors and by the addition of plant protein hydrolysates to the culture medium, In Vitro Cell. Dev. Biol. Anim. 41 (2005) 83–91.
[20] J. Suojanen, T. Salo, E. Koivunen, T. Sorsa, E. Pirila, A novel and selective membrane type-1 matrix metalloproteinase (MT1-MMP) inhibitor reduces cancer cell motility and tumor growth, Cancer Biol. Ther. 8 (2009) 2362–2370.
[21] C. van Kooten, J. Banchereau, CD40–CD40 ligand, J. Leukoc. Biol. 67 (2000) 2–17.
[22] M. Sperandio, K. Ley, The physiology and pathophysiology of P-selectin, Mod. Asp. Immunobiol. 15 (2005) 24–26.
[23] M. Asakura, M. Kitakaze, S. Takashima, Y. Liao, F. Ishikura, T. Yoshinaka, H. Ohmoto, K. Node, K. Yoshino, H. Ishiguro, H. Asanuma, S. Sanada, Y. Matsumura, H. Takeda, S. Beppu, M. Tada, M. Hori, S. Higashiyama, Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy, Nat. Med. 8 (2002) 35–40.
[24] G.J. van Mierlo, H.U. Scherer, M. Hameetman, M.E. Morgan, R. Flierman, T.W. Huizinga, R.E. Toes, Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators, J. Immunol. 180 (2008) 2747–2751.
[25] S. Rose-John, P.C. Heinrich, Soluble receptors for cytokines and growth factors: generation and biological function, Biochem. J. 300 (Pt 2) (1994) 281–290.
[26] J.H. Distler, A. Jungel, L.C. Huber, C.A. Seemayer, C.F. Reich III, R.E. Gay, B.A. Michel, A. Fontana, S. Gay, D.S. Pisetsky, O. Distler, The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2892–2897.
[27] T. Ito, N. Maki, O. Hazeki, K. Sasaki, M. Nekooki, Extracellular and transmembrane region of a podocalyxin-like protein 1 fragment identified from colon cancer cell lines, Cell Biol. Int. 31 (2007) 1518–1524.
[28] M. Hara, T. Yanagihara, T. Takada, M. Itoh, Y. Adachi, A. Yoshizumi, K. Kawasaki, T. Yamamoto, I. Kihara, Podocalyxin on the glomerular epithelial cells is preserved well in various glomerular diseases, Nephron 67 (1994) 123–124.
[29] M. Hara, T. Yanagihara, Y. Hirayama, S. Ogasawara, H. Kurosawa, S. Sekine, I. Kihara, Podocyte membrane vesicles in urine originate from tip vesiculation of podocyte microvilli, Hum. Pathol. 41 (2010) 1265–1275.
[30] K. Al-Nedawi, B. Meehan, J. Micallef, V. Lhotak, L. May, A. Guha, J. Rak, Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells, Nat. Cell Biol. 10 (2008) 619–624.
[31] K. Al-Nedawi, B. Meehan, R.S. Kerbel, A.C. Allison, J. Rak, Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3794–3799.