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Control of cell adhesion and migration by podocalyxin. Implication of Rac1 and Cdc42

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ABSTRACT

Podocalyxin (PODXL) is a type I membrane sialomucin, originally described in the epithelial cells (podocytes) of kidney glomeruli. PODXL is also found in extra-renal tissues and in certain aggressive tumors, but its precise pathophysiological role is unknown. Expression of PODXL in CHO cells enhances their adhesive, migratory and cell–cell interactive properties in a selection and integrin-dependent manner. We aimed at defining the PODXL domains responsible for those cell responses. For this purpose we have analyzed the cell adhesion/migration responses to deletion mutants of human PODXL, and the correlation with the activities of Rac1 and Cdc42 GTPases. The results obtained indicate that integrity of the PODXL ectodomain is essential for enhancing cell adhesion but not migration, while the integrity of the cytoplasmic domain is required for both adhesion and migration. Deletion of the carboxy-terminal DTHL domain (PODXL-ΔDTHL) limited only cell adhesion. The activities of Rac1 and Cdc42 GTPases parallel the PODXL-induced variations in cell adhesion and migration. Moreover, silencing the *rac1* gene virtually abolished the effect of PODXL in enhancing cell adhesion.

1. Introduction

Podocalyxin (PODXL) is a type I membrane protein of the CD34 family originally described at the luminal side of the epithelial cells of renal glomeruli (podocytes) [1]. The PODXL peptide has a molecular mass of 45 kDa that after extensive posttranslational processing results in a highly sulfated and sialylated [2,3] mature protein of 140–200 kDa [4,5]. A loss of the structural organization of the podocytes, "effacement" [6,7], and PODXL excretion in the urine are considered markers for the detection and/or progression of kidney inflammatory diseases [8,9]. PODXL appears in the glomerular podocytes at the same time as the opening of the intercellular spaces [10] and disappearance of the tight junctions [11]. PODXL-null mice die of anuria a few hours after birth [12]. Based on these observations, the proposal has been made that PODXL would act as an anti-adhesive force in the kidney glomeruli [13], contributing to maintain open the podocyte interdigitations, "urinary slits" [3].

PODXL has also been found in vascular endothelium, lung, brain, multipotent hematopoietic precursors, megakaryocytes and platelets [4,14,15], as well as in several types of tumors in which the expression levels appear to correlate with the metastatic capacity [16–18]. However, unlike the role of PODXL in the renal homeostasis, almost nothing is known about its physiological role in extra-renal tissues. We have previously reported that expression of human PODXL in CHO cells enhances their adhesion, migration and cellular interaction [19,20]. Moreover, the restricted ablation of the *podxl* gene in megakaryocytes added further evidence in support of PODXL as a proadhesive molecule [21]. In high endothelial venules (HEV), PODXL binds L-selection from circulating leucocytes and interact with integrins [22]. In contrast with CHO cells, PODXL has been reported to confer cell anti-adhesive properties to canine kidney and other cells [23–26]. How PODXL could exert either pro-adhesive or anti-adhesive effects, is a subject of debate. It should be noted that the function of PODXL could be influenced by the presence of extracellular soluble sPODXL [27].

The present work aimed at analyzing the functional properties of CHO cells stably expressing amino- or carboxy-terminal deletion mutants of PODXL and the correlation with the activities of the actin cytoskeletal organizers Rac1 and Cdc42 GTPases. The results indicate that integrity of the PODXL ectodomain is essential for enhancing cell adhesion but not cell migration. The state of activation of Rac1 and Cdc42 parallels the PODXL-induced variations in adhesion and migration. Moreover, silencing the *rac1* gene prevented the effects of PODXL. Deletion of the entire

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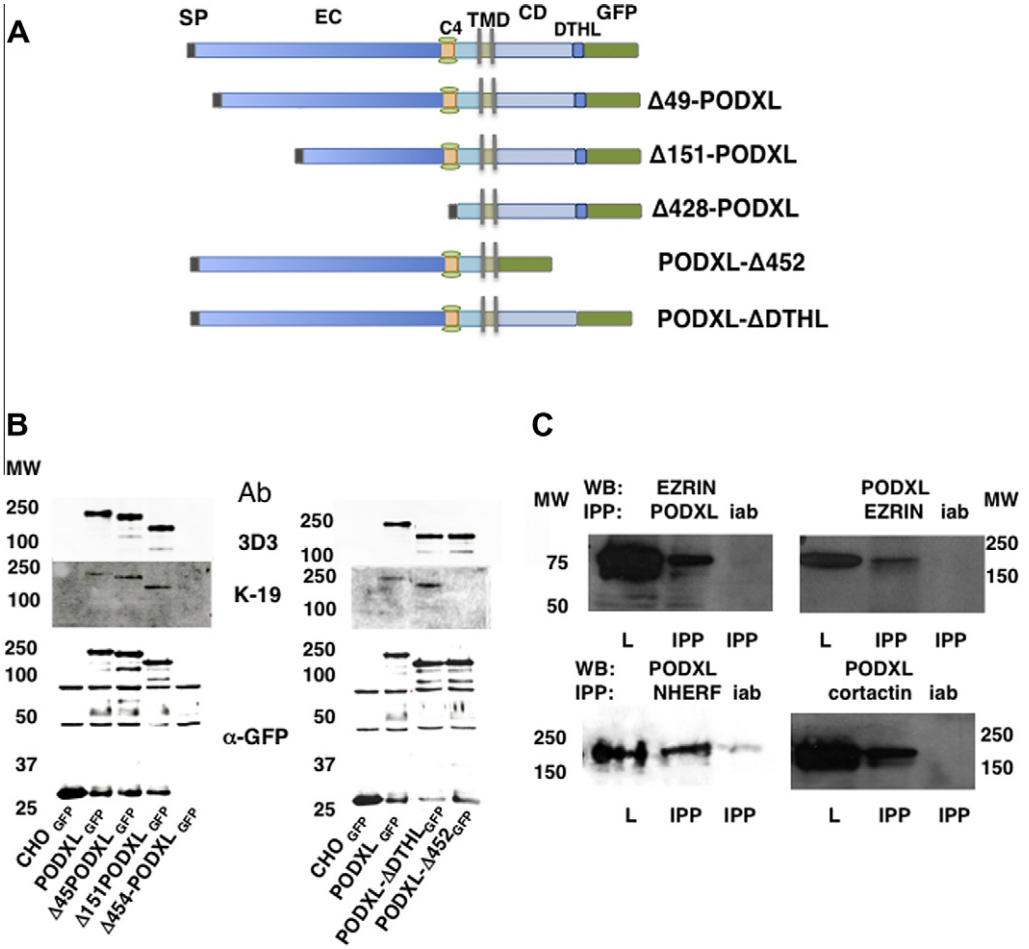

Fig. 1. Characterization of PODXL deletion mutants. (A) Scheme of $PODXL_{GFP}$ and location of deletion. *Abbreviations:* SP, signal peptide; EC, ectodomain; C4, cysteine domain; TMD, transmembrane domain; CD, cytosolic domain; GFP, green flurorescence protein; (B) Western blot identification of $PODXL_{GFP}$ and its ectodomain (left panel) and cytosolic domain (right panel) deletion mutants. The experiment was performed using the indicated antibodies as described in Methods. (C) Co-precipitation of PODXL-GFP with ezrin, NHERF-1 or cortactin in CHO cells. The experimental procedures are described in Methods. IPP, stands for immunoprecipitation antibody or immunoprecipitate; L, lysate; WB, Western blot detection antibody, iAb, mouse negative control for immunoprecipitation.

cytosolic tail of PODXL (PODXL-Δ452) virtually abolished the enhancing effects on cell adhesion and migration. Deletion of the last four carboxy-terminal residues (Aspartate-Threonine-Histidine-Leucine) (PODXL-ΔDTHL) restricted cell adhesion, but not migration. The activation of Rac1 and Cdc42 induced by PODXL was fully prevented by deletion of the cytosolic tail (PODXL-Δ452) and diminished by deletion of the DTHL domain (PODXL-ΔDTHL).

2. Material and methods

2.1. Materials

The antibodies used were: mouse anti-podocalyxin (3D3), goat anti-cytosolic domain of podocalyxin (K19), rabbit polyclonal anti-NHERF-1 (H-100), and anti-GFP (FL), mouse monoclonal anti-ezrin (3C2), anti-cortactin (F-9), and normal mouse *IgG*-AC (negative control for immunoprecipitation), all of them obtained from Santa Cruz Biotechnology. The endonuclease prepared siRNA, targeting mouse Rac1 was obtained from Sigma–Aldrich.

2.2. Cell lines and culture conditions

The cell lines stably expressing GFP, PODXL bearing a GFP tag either at the carboxy terminus (PODXL-GFP) or the amino-terminus (GFP-PODXL), or PODXL deletion mutants, prepared as previously described [19,20,27], were grown in DMEM-HT medium (Gibco) supplemented with 10% bovine fetal serum, 100 U/mL penicillin-G (Gibco) and 250 μg/mL streptomycin (Gibco), at 37 °C and 5% CO_2.

2.3. Western blotting and immunoprecipitation

Cell lysates from the different cell lines were prepared in modified RIPA buffer (50 mM pH 7.4 Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl) supplemented with 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 after overnight incubation with anti-PODXL or anti-GFP moAbs at 4 °C temperature, followed by HRP-conjugated secondary goat anti-mouse IgG. Blots were developed using an enhanced chemiluminescence's detection system (ECL). The immunoprecipitation experiments were performed using protein-G Sepharose beads (Pharmacia).

2.4. Preparation of PODXL mutants

To prepare PODXL deletion mutants with the signal peptide, we first prepared an expression vector coding a fusion protein contain-

ing the PODXL signal peptide and GFP. For this purpose the signal peptide sequence was amplified by PCR from the expression vector human PODXL cDNA-pcDNA3 [19,20], using oligonucleotides 977-1007-Kozak ATG (5′-CCC CGC GGC GAC GCC ACC ATG GGC TGC GCG-3′) and 1109-1079 (5′-CGG CGA GGA TCC CGA CGG CAG CAG CGG CGG-3′), containing restriction sites SacII and BamHI, respectively. The PCR product was cloned in the PCR 2.1-TOPO vector, and a 178-bp fragment containing the PODXL signal peptide was obtained by digestion with Hind III and Xba I, purified and, then, digested with SacII and BamH1, and cloned in the pEGFP-N1 vector (PS-PODXL-GFP vector).

In a second step, cDNA fragments corresponding to each deletion mutants were obtained by PCR and cloned into the pcDNA3 vector. For deletion mutants of the ectodomain (Δ49-PODXL, Δ151-PODXL and Δ428-PODXL), sense oligonucleotides 5′-TCT AAC AAA ACA GGA TCC ACT CCA GCA-3′, 5′-ACA GCT AAA CCT GGA TCC ACA AGC AGC CAG-3′ and 5′-CGC TTC AGC GGA TCC CTC ATC ATC ACC ATC-3′, respectively, and a common antisense oligonucleotide 1846-1822: 5′-CAG AGG ATC CAA GAG GTG TGT GTC-3′, all of them containing a BamH I site, were used.

The PCR products were digested with BamH I, purified and cloned in the PS-PODXL-GFP vector prepared in the first step of the procedure.

For deletion mutants of the cytoplasmic domain (PODXL-ΔDTHL and PODXL-Δ452), the sense oligonucleotide 977-1007-Kozak ATG, and antisense oligonucleotides 5′-CTA GAG GTG TGT GGA TCC CTC CTC ATC CAG-3′ and 5′-GGA GAG GCG GGA TCC GCA GCA GCC ATA GAG-3′, containing BamH I sites, were used. The PCR products were digested with SacII/BamH I and cloned in the pEGFP-N1 vector. All the constructs were verified by DNA sequencing.

2.5. Stable transfection of PODXL deletion mutants

CHO cells were transfected with the calcium phosphate technique and cells expressing PODXL were selected using G-418 and purified by subsequent rounds of cell sorting. The degree of expression of the different constructs was verified by flow cytometry and Western blot using anti-PODXL and anti-GFP Abs (Fig. 1).

2.6. Cell adhesion assay

Cell adhesion experiments were carried out as previously described [20]. Briefly, 96-well flat-bottomed plates were coated with 10 μg/mL fibronectin in PBS for 2 h at 37 °C and blocked with 200 μL of 1% bovine serum albumin (BSA). CHO cells growing in DMEM containing 10% fetal bovine serum were starved for 24 h, harvested by EDTA treatment, collected by centrifugation and suspended in serum-free DMEM with 0.1% BSA and 0.1% glucose. Then, 100 μL of 75×10^3 cells/well were plated in triplicate and incubated for the indicated periods of time at 37 °C. Non-adherent cells were removed by careful washes with PBS and the degree of adherence and spreading analyzed at a magnification of 10× with a phase-contrast Olympus microscope IX-50, micrographs taken with a digital camera Olympus DP-70 and the number of adhered cells blind counted.

2.7. Wound healing assay

The directional migration of cells expressing PODXL-GFP and the deletion mutants was determined by a "wound healing" assay. Cells (2×10^6) were seeded in DMEM medium with 10% FCS in 6-well fibronectin-coated culture plates. Once the cells adhered and spread, a scrape wound was created with a P10 pipette tip pushed perpendicularly through the monolayer. The plates were washed twice with PBS, incubated in full medium, and photographed immediately (0 h). After different times of incubation the migration of cells across the wound was evaluated by phase contrast microscopy. Cell migration was quantified by measuring the distance between the wound edges before and after migration at distinct positions, and the results were expressed as the % of wound closure.

2.8. Rac1 and Cdc42 activation and silencing of Rac1

The activation of Rac1 and Cdc42 in cell lysates was performed with the Rac1 activation assay kit from Millipore. To silence Rac1 we used either 6 or 24 pmol of siRNA targeting *Rac1* (Sigma Mission esiRNA) for 12-well plates or 60 mm plates, respectively, using the Mission non-target shRNA control vector as negative control. The siRNA was transfected with Turbofect siRNA reagent (Fermentas) according to the protocol of the manufacturer for adherent cells.

3. Results and discussion

The deletions of PODXL generated for this study, represented in the scheme of Fig. 1, were as follows. The signal peptide (residues 1–22) was included in all constructs. Δ49-PODXL: Deletes residues 23–49, both included, eliminating two potential N-glycosylation

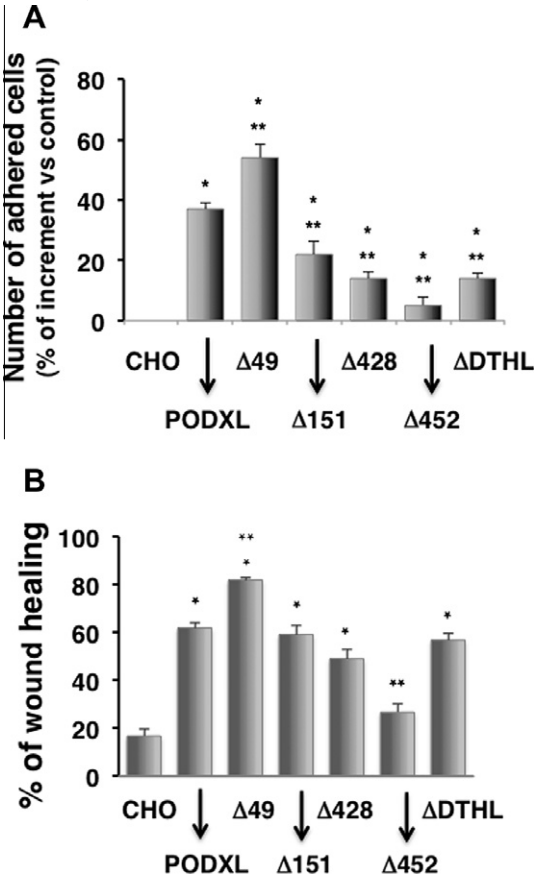

Fig. 2. Adhesion and migration of CHO cells, expressing PODXL-GFP or its deletion mutants, onto fibronectin-coated plates. (A) Cell adhesion. The results are average values of three different experiments performed in duplicate, as described in Methods, at 20 min after cell seeding. The values represent the percentage of increase over the CHO-GFP cells. (B) Cell migration determined by the wound-healing assay as described in Section 2. The results are average values of three different experiments at 12 h after seeding. By paired t test: $^*P < 0.05$ versus CHO-GFP; $^{**}P < 0.05$ versus PODXL-GFP.

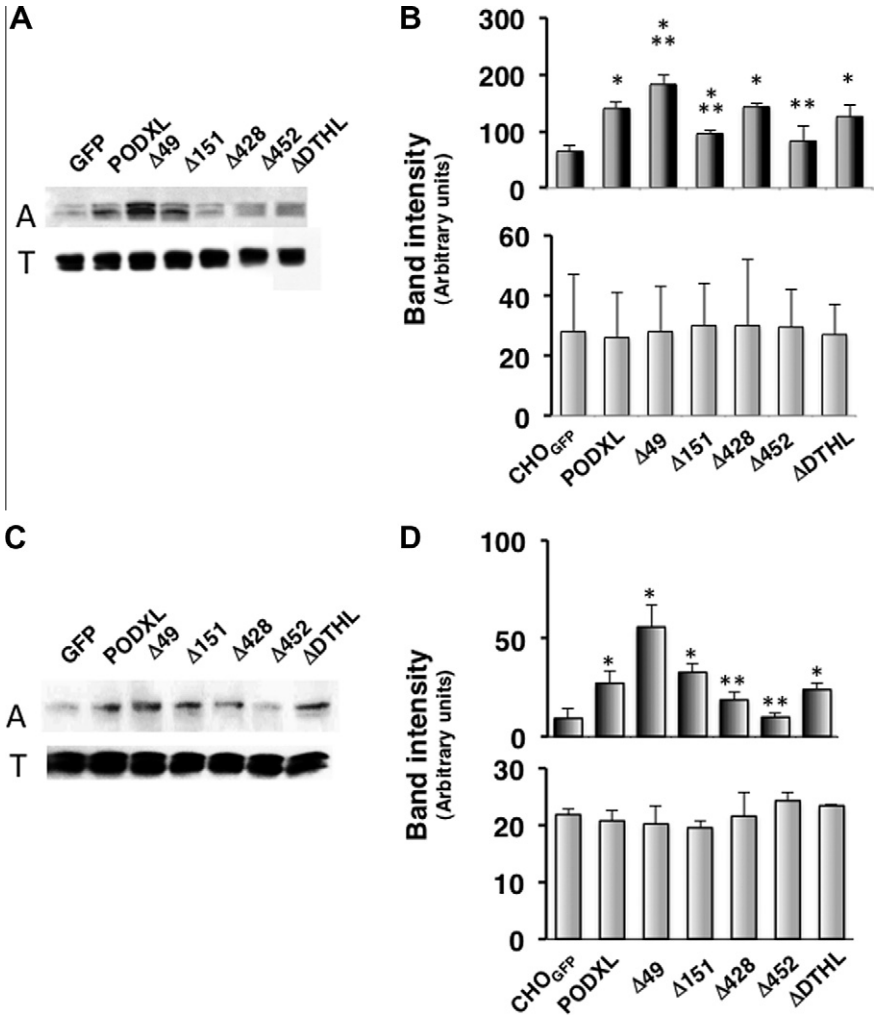

Fig. 3. Activity of Rac1 and Cdc42 in CHO cells expressing PODXL-GFP or its deletion mutants. (A) Representative Western blot of active (A) and total (T) Rac1. (B) Average values of active (upper) and total (lower) Rac1 of 6 experiments. (C) Representative Western blot of active (A) and total (T) Cdc42. (D) Average values of active (upper) and total (lower) Cdc42 of 6 experiments. By paired t test: $^{*}P < 0.05$ versus CHO-GFP; $^{**}P < 0.05$ versus PODXL-GFP.

sites (residues 33 and 43). Δ151-PODXL: Deletes residues 23–151, both included. This mutant lacks four potential N-glycosylation sites (residues 33, 43, 104 and 144) and two potential glucose-aminoglycan binding sites (102–104 and 115–117). Δ428-PODXL: Deletion of residues 23–428, both included, losing five potential N-glycosylation sites (residues 33, 43, 104, 144 and 322), four potential glucose–aminoglycan binding sites (102–104, 115–117, 160–162 and 206–208) and four cysteines (aa 314, 337, 351 and 367) postulated to form disulfide bridges [5]. PODXL-Δ452: The cytosolic tail was deleted from residue 452. PODXL-ΔDTHL: This mutation removes the last four carboxy-terminal residues Aspartate-Threonine-Histidine-Leucine (DTHL).

The levels of expression of the different mutant forms of PODXL were assessed by Western blot of cell lysates (Fig. 1B). PODXL-GFP expressed in CHO cells co-immunoprecipitates with ezrin and NHERF-1 [23,28] (Fig. 2A) indicating a similar function than the native PODXL expressed in kidney cells. In agreement with a previous report [29], PODXL in CHO cells has also been reported to interact with cortactin, as it was detected in anti-cortactin immunoprecipitates (Fig. 1C, lower right panel). We have also found cortactin in anti-PODXL immunoprecipitates (Fig. 1C lower left panel).

Fig. 2A represents the number of cells expressing either PODXL-GFP or PODXL-mutants adhered onto fibronectin-coated plates. In agreement with previous reports [19,20], PODXL enhanced the basal rate of adhesion of cells by 40–50%. Deletion of the first 49 amino terminal residues of PODXL (Δ49-PODXL) further enhanced the adhesion of cells, but the stimulating effect on cell adhesion decreased progressively as the length of the deleted fragment increased up to 151 or 428 residues (Δ151-PODXL and Δ428-PODXL, respectively.

We further analyzed the effect of PODXL deletion mutants on cell migration by the "wound-healing assay" (Fig. 2B). As with cell adhesion, CHO-Δ49-PODXL cells migrated even faster than cells expressing intact PODXL, but this effect disappeared in cells expressing either Δ151-PODXL or Δ428-PODXL, that showed similar rates of migration than CHO-PODXL cells, indicating that integrity of the ectodomain is not essential for PODXL to enhance cell migration. Dissociation of the PODXL effects on cell adhesion and migration has been previously observed by Hsu et al. in kidney cells [23]. These data suggest that interaction of the surface exposed PODXL with external agents is essential to either generate or transmit outside-in signaling controlling cell adherence. These data agrees with previous work in which deficiently sialylated CHO-PODXL cells failed to enhance cell adhesion [20].

Deletion of the entire cytosolic tail of PODXL (PODXL-Δ452) virtually abolished the PODXL effect on adhesion, whereas deletion of the carboxy-terminal DTHL domain (PODXL-ΔDTHL) reduced the

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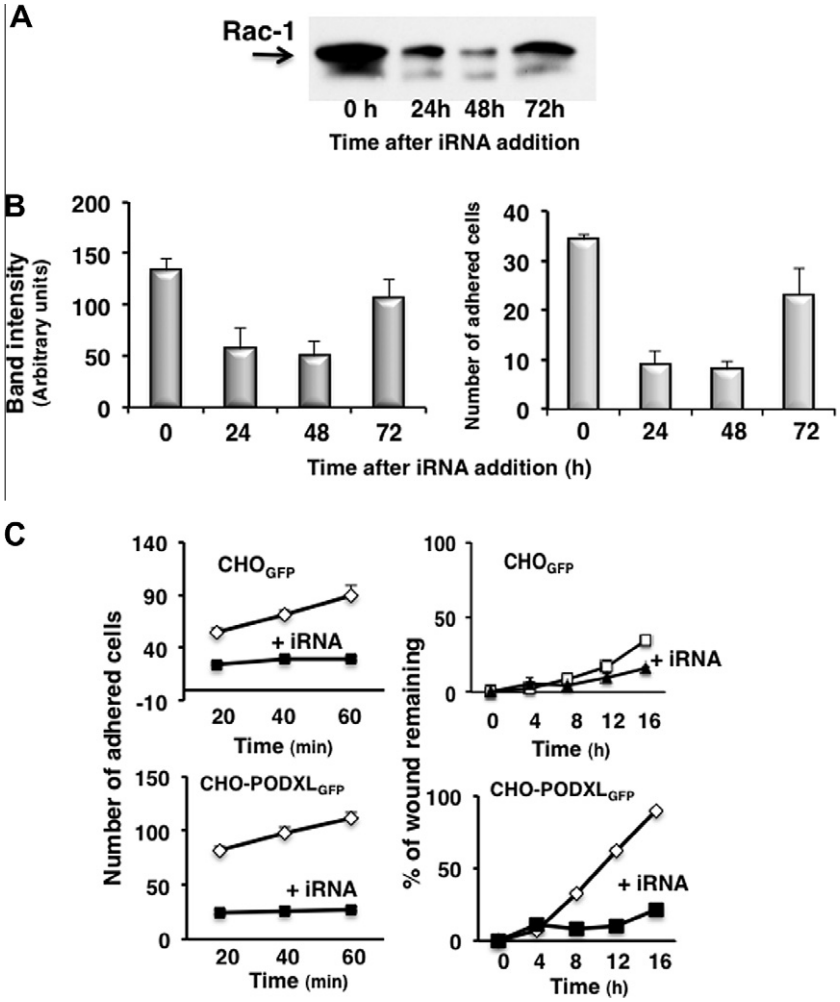

Fig. 4. Suppression of *rac1* gene diminished the effect of PODXL in enhancing adhesion and migration of CHO cells. (A) Representative Western blot analysis of Rac1 expression at the indicated times after iRNA transfection. (B) Left panel: quantification of the results of three different experiments performed as in (A). Right panel: adhesion of PODXL-GFP cells at different times after iRNA addition. The experiment was performed as described in Fig. 2. The results are mean values of three different experiments and the vertical bars the standard error of the mean. (C) Time course of adhesion (left panel) and migration (right panel) capacities of CHO cells expressing GFP or PODXL-GFP after 24-h incubation in the presence or in the absence of iRNA.

PODXL effect (Fig. 2A). The observation that the terminal DTHL domain is needed for the PODXL effect on adhesion implies that it is operative despite bearing the carboxy-terminus GFP tag. Moreover, as previously reported [27] no detectable differences in enhancing cell adhesion could be found when the GFP tag was linked to the amino-terminus of PODXL (GFP-PODXL) (Fig. S1). These observations suggest that signaling pathways other than those mediated by the interaction of the DTHL domain with NHERF-1 and ERM complex must be mediating the PODXL action on cell migration. Cortactin could a candidate for this interaction, that regulates cell motility through binding to Arp 2/3 [30], and reacts with PODXL [29]. In fact, we found cortactin in PODXL immunoprecipitates from CHO-PODXL cell lysates (Fig. 1). Since ezrin may interact with PODXL not involving the DTHL domain [31], perhaps it should also be considered as a potential factor involved in controlling the PODXL action.

The participation of NHERF proteins and ERM complex in the action of PODXL implies the involvement of cytoskeletal organizers Rac1 and Cdc42, members of the Rho family of small GTPases [23]. Thus, we studied the activities of Rac1 and Cdc42 in cells transfected with the different PODXL mutants. Expression of PODXL-GFP was accompanied by activation of both Rac1 and Cdc42 above the control values (CHO-GFP) (Fig. 3). The Δ49-PODXL mutant, that showed a marked effect in enhancing cell adhesion and migration (Fig. 2), did also show a significant increase in the Rac1 and Cdc42 activities. To note, the enhancing functional effects of removing the amino-terminus PODXL is consonant with the positive association of variants of PODXL carrying in-frame deletions of 2, 4 or 6 amino acids at positions 23–24, 23–26 or 23–28, respectively, with the risk of both prostate cancer and tumor aggressiveness [18].

The Δ151-PODXL and Δ428-PODXL mutants produced an activation of GTPases, although to a much lesser extent than PODXL-GFP or Δ49-PODXL. PODXL-Δ452 and PODXL-ΔDTHL produced changes in Rac1 and Cdc42 activation parallel to the variations on cell adhesion and migration. To note, PODXL-ΔDTHL produced a significant activation of Rac1 and Cdc42, although the activation was smaller than that produced by intact PODXL.

To assess the participation of Rac1 in the enhancing effects of PODXL on cell adhesion and migration, we knocked down the *rac1* gene by transfecting Rac1-iRNA. The time course of Rac1 knockdown is shown in Fig. 4A and B. The maximum effect of iRNA was obtained at 48 h and partially reversed at 72 h. The enhancing effects of PODXL in cell adhesion and migration were prevented 24 h after the administration of Rac1-iRNA (Fig. 4C). The effect of PODXL on cell adhesion parallels Rac1 knockdown, recovering after 72 h (Fig. 4B). Similar observations were obtained by using other PODXL deletion mutants.

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The dependence of PODXL action on integrins [20] agrees with the postulate that activation of GTPases would contribute to integrin clustering and positioning within the cell adhesion contacts. However, the present work does not explain how PODXL could enhance the proposed integrin-mediated activation of small GTPases. Whatever the signaling mechanism, the lack of effects of PODXL in the absence of its cytosolic tail implies a participation of the intracellular tail of PODXL in the signaling path.

The present results seem to be in conflict with the postulated anti-adherent properties of PODXL in renal podocytes [32]. The close relationship between the PODXL effect in enhancing cell adhesion and the activity of the small GTPases rules out any possible artifact in the PODXL effects. Moreover, silencing the *rac1* gene abolished the PODXL effect in enhancing cell adhesion/migration. A differential distribution of intracellular regulatory proteins [24] could be of importance to explain the distinct effects of PODXL in different experimental models.

To summarize, the use of deletion mutants suggest that a domain encompassed by residues 49 to $\geqslant$151 determines the enhancing effect of PODXL on cell adhesion, suggesting the operation of an outside-in signal. In contrast, cell migration could be detected even in the absence of the entire ectodomain, suggesting that interaction of the intracellular domain of PODXL with intracellular regulatory proteins suffices to enhance cell migration. The finding of parallel changes in cell adhesion/migration and Rac1 and Cdc42 activities and the effect of *rac1* suppression on the PODXL effects, suggests that reorganization of the cytoskeletal network mediated by GTPases plays a primary role in the cellular responses to PODXL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.112.

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