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Ezrin is a specific and direct target of protein tyrosine phosphatase PRL-3

Eleonora Forte [a,b], Laura Orsatti [a], Fabio Talamo [a], Gaetano Barbato [a], Raffaele De Francesco [a], Licia Tomei [a,*]

[a] *IRBM P. Angeletti, via Pontina km 30,600, 00040 Pomezia, Rome, Italy*
[b] *Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06126 Perugia, Italy*

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Abstract

Phosphatase of Regenerating Liver-3 (PRL-3) is a small protein tyrosine phosphatase considered an appealing therapeutic cancer target due to its involvement in metastatic progression. However, despite its importance, the direct molecular targets of PRL-3 action are not yet known. Here we report the identification of Ezrin as a specific and direct cellular substrate of PRL-3. In HCT116 colon cancer cell line, Ezrin was identified among the cellular proteins whose phosphorylation level decreased upon ectopic over-expression of wtPRL-3 but not of catalytically inactive PRL-3 mutants. Although PRL-3 over-expression in HCT116 cells appeared to affect Ezrin phosphorylation status at both tyrosine residues and Thr567, suppression of the endogenous protein by RNA interference pointed to Ezrin-Thr567 as the residue primarily affected by PRL-3 action. *In vitro* dephosphorylation assays suggested Ezrin-Thr567 as a direct substrate of PRL-3 also proving this enzyme as belonging to the dual specificity phosphatase family. Furthermore, the same effect on levels of pThr567, but not on pTyr residues, was observed in endothelial cells pointing to Ezrin-pThr567 dephosphorylation as a mean through which PRL-3 exerts its function in promoting tumor progression as well as in the establishment of the new vasculature needed for tumor survival and expansion.

Keywords: PTP; Substrate identification; Phosphorylation; ERM proteins; RNA interference

1. Introduction

Modulation of the phosphorylation state of proteins by the coordinated action of kinases and phosphatases is a key regulatory mechanism of many eukaryotic cellular processes. The protein tyrosine phosphatase (PTP) superfamily of phosphatases includes a large group of enzymes important for the regulation of a wide variety of cellular mechanisms including signal transduction, cell cycle, differentiation, cellular transformation as well as adhesion and motility [1,2]. Among them, increasing evidence is accumulating suggesting that PRL tyrosine phosphatases (*P*hosphatase of *R*egenerating *L*iver-1, -2 and -3) play a potentially pathogenic role in human malignancy and, above all, PRL-3 could be considered an appealing therapeutic cancer target due to its involvement in processes of cell motility and invasion determining metastatic progression [3]. Among normal adult human tissues, PRL-3 mRNA is expressed primarily in skeletal muscle and heart, with lower expression levels in pancreas, spleen, lung and testis [4,5] but little is currently known about its physiological role in these tissues. In mouse, the presence of PRL-3 in the differentiated epithelial cells of the villus – but not in the proliferating cryptic cells – suggests that PRL-3 may contribute to the differentiation of epithelial cells in the small intestine [6]. In addition, a role in cardiac functions is indicated by the observation that PRL-3 appears to be involved in the modulation of intracellular calcium mobilization in response to Angiotensin II [4].

In the last few years, evidence has accumulated for the association of PRL-3 with oncogenic states and several indications have linked its expression to human colon cancer progression and metastasis. The first data came from the analysis of global gene-expression profiling of metastatic colorectal cancers (CRC) which showed that PRL-3 is selectively over-expressed in metastatic CRC cells with lower levels in non-metastatic tumors and normal colorectal epithelium [7]. PRL-3

* Corresponding author. Tel.: +39 06 91093230; fax: +39 06 91093654.
E-mail address: licia_tomei@merck.com (L. Tomei).

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expression appeared to be elevated in nearly all CRC metastatic lesions irrespective of the target organ [8,9]. PRL-3 expression has also been reported in a Hodgkin's lymphoma cell line [10], melanomas, liver, ovarian, breast and gastric cancers where its presence seems to have an important role in the acquisition of metastatic potential of tumor cells [11,12]. PRL-3 might also be considered as a good molecular marker of tumor aggressiveness due to its expression in primary cancers as predictor of metastasis [13,14].

Consistent with a role of PRL-3 over-expression in metastasis, its ectopic expression in different cell types is correlated with the induction of metastatic phenotypes, such as invasion and motility capabilities [11,15,16]. On the contrary, its transient down-regulation by RNA interference has an opposite effect [9]. These data are corroborated by *in vivo* data where PRL-3 over-expression promotes experimental metastasis in mice [11,15,16] while its down-regulation in human colon cancer cells abrogates their metastatic potential [9,17]. A possible role of PRL-3 in tumor-related angiogenesis has also been suggested by data correlating PRL-3 expression with the genesis of new vasculature [18] and by evidences showing that some metastatic tumors sprout and grow into well-established blood vessels [16].

Recently, Fiordalisi et al. [19] demonstrated that PRL-3 modulates small GTPases of the Rho family and activates the serum response element (SRE) transcription factor in a Rho-dependent manner, suggesting not only a direct involvement of this phosphatase in cytoskeleton modeling but also a role in the transcriptional regulation of target genes. Liang et al. [20] found that PRL-3 ectopic expression causes a down-regulation of Csk level leading to a more active form of Src kinase. This activation triggers several signal pathways culminating in the phosphorylation Erk1/2, STAT3, and $p130^{Cas}$ which are responsible for cell proliferation and invasion promotion. However, the only PRL-3 interacting protein currently known is integrin α1 [21] and direct targets of PRL-3 action have not been identified yet.

Here we report the identification of Ezrin as a specific target of PRL-3: Ezrin emerged among the cellular proteins whose phosphorylation level changed upon either ectopic over-expression of the PRL-3 protein or down-regulation of the endogenous PRL-3 mRNA by RNA interference. Although PRL-3 over-expression in the HCT116 colon cancer cell line appeared to affect Ezrin phosphorylation status at both tyrosine residues and Thr567, silencing experiment, combined to *in vitro* dephosphorylation assays, suggested that PRL-3 specifically acts on Ezrin-pThr567. Furthermore, the same effect on pThr567, but not on pTyr residues, was observed in endothelial cells, where expression of endogenous PRL-3 protein was enhanced by PMA treatment, pointing to pThr567 as the specific and possibly direct target of PRL-3 on Ezrin.

2. Materials and methods

2.1. Cells

HCT116 from ATCC were cultured at 37 °C/5%CO_2 in McCoy's medium (Invitrogen), supplemented with 10%FCS, antibiotics (streptomycin and penicillin 100 U/ml) and L-Glutamine 2 mM. HUVEC (Human Umbilical Vein Endothelial Cells pooled from Lonza) were grown in EGM bullet kit (Lonza).

2.2. Antibodies

Anti-human Ezrin (clone 3C12), α-GAPDH and α-tubulin were obtained from Sigma. Anti-Phosphotyrosine (clone 4G10) and α-Phosphotyrosine (4G10) agarose-conjugate were from Upstate. Anti-PhosphoEzrin(Thr567) and PhosphoEzrin(Tyr353) were from Cell Signaling. Anti-PhosphoEzrin(Tyr145) was from Santa-Cruz Ltd. Peroxidase-conjugated secondary antibodies were purchased from Sigma. A polyclonal antibody directed against PRL-3 was obtained in rabbit using as antigen the PRL-3 protein lacking the C-terminal 4 residues expressed in *E. coli*. Anti-Flag M2 Affinity gel freezer-Safe was from Sigma.

2.3. Plasmids

The wild-type PRL-3 coding sequence (NM 032611) was amplified by PCR from a skeletal muscle cDNA library (Clontech) and inserted into BamHI and EcoRI sites of the pcDNA3 vector (Invitrogen) giving rise to the construct pCDwtPRL-3. The constructs pCDPRL-3(D72A) and pCDPRL-3(D72A/C104S) were derived by site directed mutagenesis from pCDwtPRL-3 and contained the following mutations: pCDPRL-3(D72A), replacement of GAT codon for D72 with GCT (coding for A); pCDPRL-3(D72A/C104S), replacement of GAT codon for D72 with GCT (coding for A) and replacement of TGC for C104 with TCC (coding for S). For construction of pCD-FlagPRL-3, a DNA sequence coding for the Flag epitope (MDYKDDDDK) followed by a GSGS link was inserted by PCR at the N-terminus of the PRL-3 sequence in the plasmid pCDwtPRL-3. Constructs were confirmed by DNA sequencing.

2.4. DNA transfection

HCT116 cells were transfected with pCD-PRL-3 plasmids or empty vector using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were incubated 4 h at 37 °C/5%CO_2 after which time cells were detached by Trypsin-EDTA treatment, viable cells were counted with Guava and seeded for 24, 48 and 72 h incubation (37 °C/5%CO_2). Before collection, cells were treated with Sodium orthoVanadate and H_2O_2 0.1 mM for 30 min at 37 °C, with the exception of those transfected with the pCD-FlagPRL-3 plasmid. Cells transfected for the expression of wtPRL-3 and mutant proteins were suspended in lysis buffer (Tris 20 mM, pH 7.5, NaCl 100 mM, Triton 1%, Glycerol 10%, EDTA 1 mM, Okadaic acid1 μM (Calbiochem), Sodium Pyrophosphate 10 mM, NaF 20 mM, Iodoacetic acid 5 mM (Sigma), DTT 1 mM, Sodium orthoVanadate 0.1 mM, Complete (Roche)) and incubated 30 min on ice. Cells over-expressing FlagPRL-3 were lysed in Tris 20 mM, pH 7.5, NaCl 100 mM, Triton 1%, Glycerol 10%, EDTA 1 mM, TCEP 1mM, DPTA 1 mM, and Complete. Protein concentrations in the extracts were determined by Bradford method assay (BIO-RAD) and the presence of PRL-3 confirmed by western blot using horseradish peroxidase-conjugated secondary antibodies (Pierce) and ECL detection system (Amersham).

2.5. Mono-dimensional gel electrophoresis and mass spectrometry

Equal amounts of total protein extracts form mock- or PRL-3-transfected cells were resolved by SDS-PAGE. The gels were initially incubated with Pro-Q Diamond (Molecular Probes) for phospho-protein staining followed by destaining and incubation with SYPRO Ruby stain (Molecular Probe) for total protein visualization, as recommended by the manufacturer. Detection was performed on a Thyphoon 9410 (Amersham) using excitation/emission wavelengths as recommended by the manufacturer. For the identification of Ezrin, protein bands were excised from mono-dimensional gel using the ProXcision spot picker (Perkin Elmer Instruments, Shelton, CT, USA). Excised spots were digested with Multiprobe liquid handler (Perkin Elmer Instruments). Gel plugs were first washed with 100 mM ammonium bicarbonate for 5 min, 100 mM ammonium bicarbonate/acetonitrile 50/50 for 5 min and pure acetonitrile for a further 5 min. 50 μl of 12 ng/μl trypsin (Promega, Madison, WI) were added to each gel piece and after 45 min at 4 °C the trypsin solution was replaced with 50 mM ammonium bicarbonate for 12 h at 37 °C. Tryptic peptides were then extracted from the gel with 5% formic acid and dried by vacuum centrifugation. Peptides were resuspended in 0.5% acetic acid and analyzed by LC-IT-MSMS.

For mass spectometry, an LCQ DECA XP-Plus ion trap mass spectrometer (Thermo Electron, San José, CA) equipped with an in-house built micro-

electrospray ion source coupled with a Surveyor HPLC (Thermo Electron, San José, CA) was used. Samples were loaded in an in-house packed pre-column (C18 resin, 5 μm particle size) placed before a C18 column packed in the sprayer. Peptides were separated and eluted from the column with a 0.5% acetic acid/acetonitrile gradient (from 0.5% acetic acid/acetonitrile 98/2 to 0.5% acetic acid/acetonitrile 50/50 in 30 min, flow rate 1 μl/min). MS and MSMS spectra acquired in a data dependent manner were searched against the SWISS-PROT database using the TurboSequest software provided by the manufacturer.

2.6. RNA interference

For the RNA interference of human PRL-3 a specific small interfering RNA (siRNA) was used (si1: 5′-TCTCGTTTCTCTTGGACAA-3′ On Target 2′-hydroxyl, annealed and desalted duplex — Dharmacon). The following siRNAs were used as negative controls: *Renilla* luciferase: 5′-AAAAACATGCAGAAAATGCTG-3′; GL2: 5′ CGTACGCGGAATACTTCGA 3′ (Dharmacon). HCT116 cells were seeded in 6-well plates at 1.5×10^5 cells/well in complete McCoy's for 30% confluence the next day. The following day, siRNA si1 were transfected at a final concentration of 90 nM in 300 μl of Optimem Reduced Serum Medium (Invitrogen) in presence of Oligofectamine (Invitrogen). After transfection, cells were incubated 4 h at 37 °C/5%CO_2 after which time 3 ml complete medium were added and cells were further incubated for 20 h at 37 °C/5%CO_2. Cells were collected at 24 h post-transfection or medium was replaced for longer incubation time.

HUVEC were seeded in 6-well plates at 3.5×10^5 cells/well in EGM medium. After 8 h the cells were treated with 100 nM PMA (Phorbol 12-myristate 13-acetate; Sigma) in DMSO in EBM medium for 12 h at 37 °C/5%CO_2 for inducing the expression of endogenous PRL-3. siRNA si1 was added to the culture at a final concentration of 10 nM in 900 μl of Optimem without serum in presence of Lipofectamine 2000. After transfection, cells were incubated 4 h at 37 °C/5%CO_2 after which time complete medium supplemented with PMA/DMSO 100 nM was added and cells were further incubated for 20 h. Cells were collected at 24 h post-transfection or medium was replaced for longer incubation time. Unless otherwise specified, both HCT116 cells and HUVEC were treated for 30 min at 37 °C/5%CO_2 before collection with Sodium orthoVanadate (Aldrich) and H_2O_2 at 0.1 mM final concentrations.

2.7. Quantitative real time RT-PCR analyses

Purification of total RNA was performed using RNeasy Miny kit (Qiagen) following manufacturer's instruction Quantitative real time RT-PCR analyses were performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and threshold numbers observed using SDS 2.2.2 software.

The following primer set was used for RT-PCR amplification of endogenous PRL-3 mRNA: Forward 5′-AGGACGCCATCCAGTTCATC-3′; Reverse 5′-AGGTGAGCTGCTTGCTGTTA-3′; Probe 5′-CCAGAAGCGCCGCGGAGC-3′.

As internal control, Human 18S rRNA (Applied Biosystems) was used. According to the manufacturer's instruction, a master mix (50 μl) of the following reaction components was prepared to the indicated final concentration: 25 μl of 2× Universal PCR Master Mix (Applied Biosystems), 1.25 μl of 40× Multiscribe and RNase Inhibitor Mix (Applied Biosystems), 90 nM of each forward and reverse primer, 150 nM of probe, 10 μl (100 ng) of total RNA and the proper amount of RNase-free water. After an initial incubation at 48 °C for 30 min and denaturation at 95 °C for 10 min, the following cycling conditions (40 cycles) were used: denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 1 min.

2.8. PMA and TNF-α treatment of HUVEC

HUVEC were seeded in 6-well plates at 5×10^5 cells/well in EGM. The following day, 100 nM PMA/DMSO, or DMSO alone, was added to the cells in serum-free EBM2 and incubation continued for 24 h at 37 °C/5%CO_2. For TNF-α

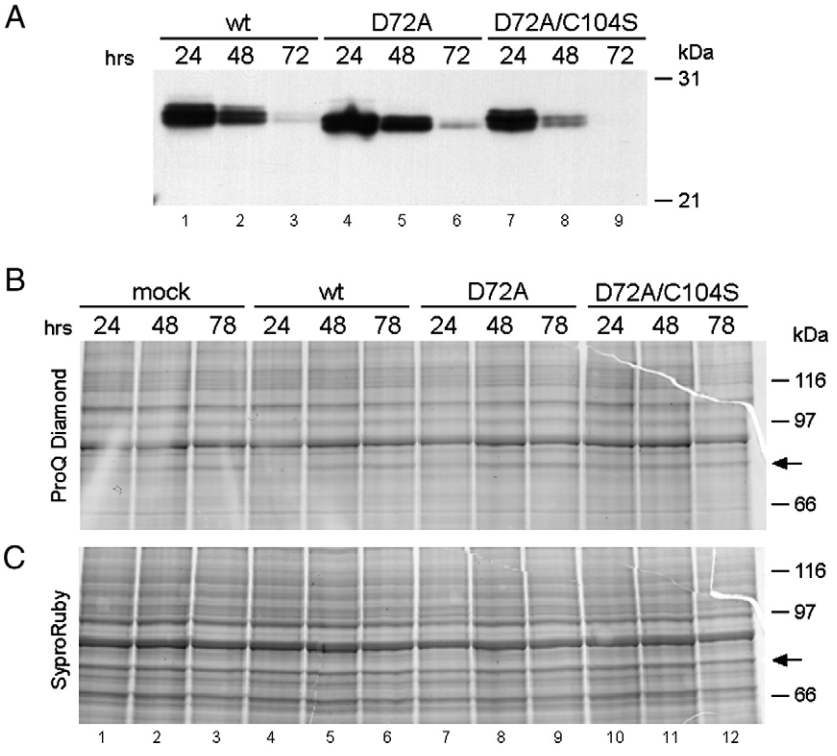

Fig. 1. The phospho-protein pattern of HCT116 cells is affected by over-expression of wtPRL-3 but not of its catalytically inactive mutants. A) Western blot analysis of wild-type (wt, lanes 1,2,3) and mutants (D72A, lanes 4,5,6; D72A/C104S, lanes 7,8,9) PRL-3 proteins in transiently transfected HCT116 cells. Total proteins extracts were prepared at 24, 48 and 72 h post-transfection and 15 μg were loaded on 12.5% SDS-PAGE. Rabbit polyclonal antibody against wtPRL-3 expressed in *E. coli* was used at 1:1000 dilutions. B) Total protein extracts were prepared from HCT116 cells mock-transfected (lanes 1,2,3) or transiently transfected with wild-type (wt, lanes 4,5,6) and mutants (D72A, lanes 7,8,9; D72A/C104S, lanes 10,11,12) PRL-3 constructs at 24, 48 and 72 h post-transfection and 40 μg were resolved on 7.5% SDS-PAGE. The gel was stained with ProQ Diamond for phospho-protein pattern analysis followed by (C) total protein staining by SyproRuby. The arrowheads in panel B and C indicate the band whose intensity in the ProQ Diamond-stained gel appears diminished upon wtPRL-3 over-expression.

treatment, cells that were or were not treated with PMA, were incubated for 1 h at 37 °C/5%CO_2 in EGM in the presence of 20 ng/ml recombinant human TNF-α (R&D Systems) or vehicle alone (PBS containing 0.1% BSA).

2.9. PTPase assays

For FlagPRL-3 immunoprecipitation, 900 μg of total extract from FlagPRL-3 or mock-transfected cells not treated with orthoVanadate were incubated overnight at 4 °C with 60 μl of Anti-Flag M2 affinity gel freezer-safe (Sigma) in lysis buffer. Protein elution was performed under native condition by competition with 100 μg/ml 3× Flag peptide (MDYKDHDGDYKDHDIDYKDDDDK; Sigma) at RT for 1 h in 180 μl Elution buffer (Tris 50 mM, pH 7, NaCl 100 mM, Triton 0.05%, Glycerol 5%, TCEP 1 mM, DPTA 1 mM). The presence of wtPRL-3 was assessed by western blot analysis and the total protein pattern of mock and FlagPRL-3 eluted extracts compared by silver-staining of SDS-gels.

The PTPase activity of the eluted FlagPRL-3 was assessed by incubating 70 μl of mock or FlagPRL-3 eluted extracts with diFMUP (6,8-difluoro-4 methylumbelliferyl phosphate) 100 μM in 80 μl final volume for 4 h at 37 °C. To each reaction, 20 μl of Enzolyte reagent (AnaSpec) was added and the amount of phosphate freed from the substrate quantified by spectrometry ($A_{620\ nm}$).

For endogenous phospho-Ezrin immunoprecipitation, 100 μg of total extracts from orthoVandate-treated HCT116 were incubated overnight at 4 °C with 30 μl of anti-PhosphoTyrosine (4G10) agarose-conjugate in lysis buffer (Tris 20 mM, pH 7.5, NaCl 100 mM, Triton 1%, Glycerol 10%, EDTA 1 mM, Okadaic acid 1 μM, Sodium Pyrophosphate 10 mM, NaF 20 mM, Iodoacetic acid 5 mM, DTT 1 mM, Sodium orthoVanadate 0.1 mM, Complete). After incubation, the agarose beads were washed three times in assay buffer (Tris 50 mM, pH 7, NaCl 100 mM, Triton 0.05%, Glycerol 5%, TCEP 1 mM, DPTA 1 mM) and incubated with 100 μl of FlagPRL3 or mock-immunoprecipitated material for 4 h at 37 °C. The levels of Ezrin phospho-tyrosines and pThr567 affected by incubation with FlagPRL-3 were analyzed by western blot, using Ezrin as internal control. Image acquisition was performed with LAS-3000 (FUJIFILM) and elaborated with Multigauge V2.2 (FUJIFILM).

3. Results

3.1. Identification of Ezrin as a cellular target of PRL-3

With the aim of identifying the PRL-3 molecular targets, we transiently over-expressed in the colon cancer cell line HCT116 the wild-type PRL-3 (wt) and the two mutant proteins PRL3-D72A (D72A) and PRL3-D72A/C104S (D72A/C104S), which respectively displayed partially or completely abolished catalytic activity, in order to observe differences in their phospho-protein patterns. PRL-3 over-expression was monitored by western blot analysis using a polyclonal antibody raised against the PRL-3 protein expressed in *E. coli*. For both the wt and mutant proteins, maximal expression was observed at 24 h post-transfection, after which time the expression level significantly decreased (Fig. 1A). The differences between the phospho-protein pattern of total protein extracts from HCT116 cells transiently transfected for the expression of wt or mutant PRL-3 proteins were evaluated after Phospho-staining of mono-dimensional SDS-PAGE gels (Fig. 1B). A clearly different phospho-protein pattern was observed in the cells over-expressing wtPRL-3 at 24 h post-transfection (Fig. 1B), time at which the expression of the ectopic protein was maximal. Particularly, phosphorylation of a band migrating as an 80 kDa protein appeared heavily affected by wtPRL-3 expression (lane 4) but not affected by expression of PRL3-D72A mutant (lane 7) and of the double mutant D72A/C104S (lane 10). Each lane of the gel contained essentially the same amount of total protein loaded, as evidenced by SyproRuby staining (Fig. 1C).

The 80 kDa protein whose phosphorylation level diminished in the PRL-3 over-expressing cells was identified by LC-IT-MSMS as corresponding to Ezrin (Swiss-Prot # P15311). PRL-3 over-expression only affected the phosphorylation state of Ezrin, but not its level of expression, which remained unchanged as also indicated by western blots probed with an Ezrin-specific antibody (Fig. 2).

Ezrin was confirmed as the protein whose phosphorylation state was the greatest affected by over-expression of PRL-3 by 2-D DIGE (2-D Difference Gel Electrophoresis) experiments combined with LC-IT-MSMS (data not shown. In 2-D gels Ezrin was resolved as a train of nine spots corresponding to differently modified forms of the protein, three of which resulted to be not phosphorylated and six phosphorylated. Upon PRL-3 over-expression, a reduction of the phosphorylated forms of Ezrin and an increase of the non-phosphorylated ones were clearly noticed (data not shown).

3.2. PRL-3 over-expression affects the phospho-tyrosine pattern of Ezrin and causes dephosphorylation of pThr567

Ezrin, together with Radixin and Moesin, constitutes the ERM (Ezrin/Radixin/Moesin) protein family whose main function is to provide a regulated bridge between cell surface receptors and

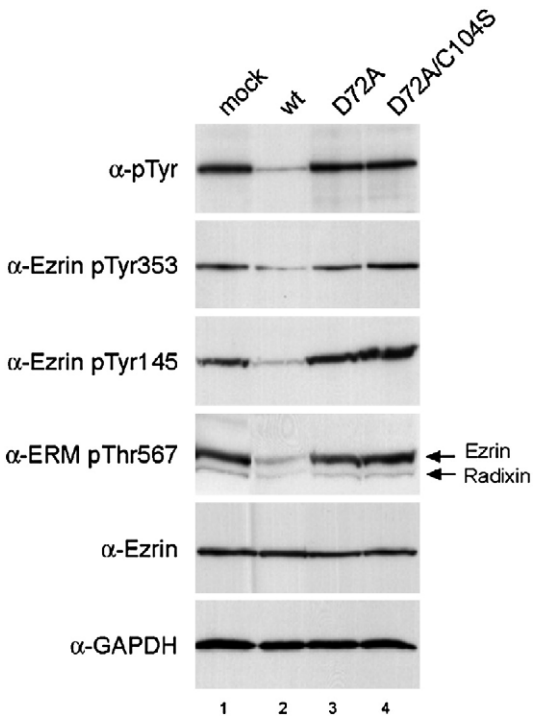

Fig. 2. PRL-3 over-expression reduces phosphorylation of Ezrin-pTyr residues and pThr567 in HCT116. Western blot analysis of Ezrin and Ezrin-phosphorylated forms in HCT116 cells transiently transfected with wild-type (wt, lane 2) and mutant PRL-3 constructs (D72A, lane 3; D72A/C104S, lane 4) in comparison to mock-transfected cells (mock, lane 1). Total protein extracts were prepared at 24 h post-transfection and 20 μg were loaded on 7.5% SDS-PAGE for probing with α-Ezrin antibody (1:1000), α-pTyr(clone 4G12) (1:2000) and α-Ezrin pTyr353 (1:1000). For probing with α-ERMpThr567 (1:300) and α-Ezrin pTyr145 (1:300) 150 μg total proteins were loaded. Immunoblots were developed by the enhanced chemioluminescence technique.

actin cytoskeleton [22]. Ezrin is activated by phosphorylation of Thr567 and the phosphorylation status of a number of Tyr and Ser residues regulates its involvement in different cellular processes [23].

We investigated whether PRL-3 acts on pTyr-Ezrin by western blot analysis comparing HCT116 cells with or without over-expression of wtPRL-3 and probing with an antibody generically recognizing pTyr residues. As evident from Fig. 2 (α-pTyr), a remarkable down-regulation of the Ezrin tyrosine-phosphorylation level was observed upon ectopic over-expression of PRL-3 (compare lanes 1 and 2).

While the clear effect on the overall phospho-tyrosine pattern of Ezrin observed is presumably caused by the dephosphorylation of several residues, the lack of commercial antibodies directed against each of the known phospho-tyrosine residues made it difficult to precisely define which residues are affected by PRL-3 action. We focused, therefore, our interest on two of the most studied pTyr residues of Ezrin, pY353 and pY145 which are both involved in several processes such as cell motility, cell spreading and proliferation [24,25]. Since PRL-3 is implicated in cell migration ([15] and E. Forte, unpublished results), both Ezrin-pY353 and pY145 might be possible targets of PRL-3. Hence, we analyzed the effect of the ectopic expression of wtPRL-3 on these residues using commercial antibodies specific for Ezrin-pY353 and pY145. As clearly shown in Fig. 2, a marked effect of wtPRL-3 over-expression (lane 2), but not of its mutants (lanes 3 and 4), on the phosphorylation state of both Ezrin-pY353 and pY145 was observed, clearly pointing to these residues as possible target sites of PRL-3.

Phosphorylation of Thr567 in ERM proteins is critical for the activation of the protein triggering the open conformation competent for actin binding and correct localization to the membrane [26]. Western blot analysis with an antibody directed against ERM-pThr567 (Fig. 2) showed that phosphorylation at T567 was weakened by wtPRL-3 over-expression (lane 2) while no effect was observed upon over-expression of the inactive protein forms (lanes 3 and 4). The antibody directed against pThr567 is not specific for Ezrin being the peptide containing Thr567 common to all the three ERM proteins. Consequently, radixin is also observed in the mono-dimensional gel system probed with this antibody (Fig. 2). However, it is interesting to note that only the phosphorylation state of Ezrin-Thr567 appeared to be affected by wtPRL-3 over-expression since the intensity of the band corresponding to radixin is almost unchanged with respect to the mock-transfected sample (Fig. 2, lanes 1 and 2). This result not only indicated Thr567 as a further target site of PRL-3 on Ezrin but also suggested PRL-3 action specifically directed against Ezrin.

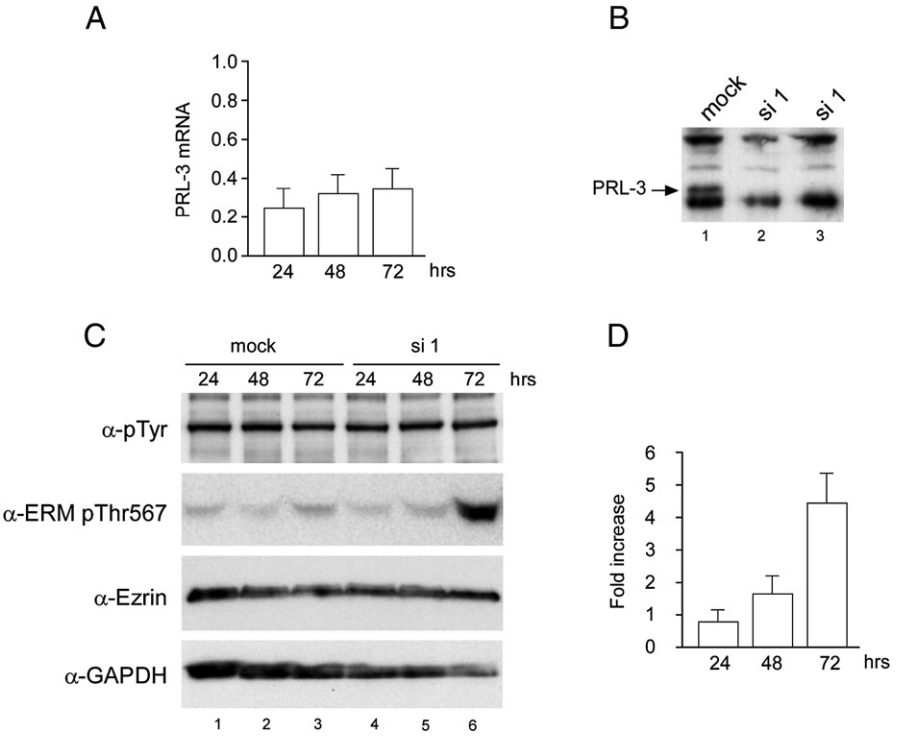

Fig. 3. Endogenous PRL-3 suppression by RNA interference in HCT116 only influences the phosphorylation state of Ezrin-pThr567. A) Effect of PRL-3 silencing on endogenous PRL-3 mRNA level at 24, 48 and 72 h post-transfection with 90 nM si1RNAs, as determined by quantitative Real Time PCR. PRL-3 mRNA amount in silenced cells is expressed relative to its level in mock-transfected cells, arbitrary set to 1. B) Western blot analysis of the endogenous PRL-3 protein in PRL-3 silenced HCT116 cells. 100 μg of total protein extracts from cells mock-transfected (lane 1) or transfected (lane 2 and 3) with the si1 RNA at 24 h (lane 2) and 72 h (lane 3) post-transfection were resolved on 12.5% SDS-PAGE probed with α-PRL-3 antibody. The arrow indicates the band corresponding to the endogenous PRL-3 protein in mock-transfected cells. C) Total protein extracts were prepared at 24, 48 and 72 h after mock-transfection (lanes 1,2,3) or PRL-3 silencing (lanes 4, 5, 6) and 20 μg were loaded on 7.5% SDS-PAGE for probing with α-Ezrin, α-pTyr (clone 4G12) and α-GAPDH (1:1000). 150 μg total proteins were loaded for probing with α-ERM-pThr567. D) Down-regulation of PRL-3 caused a 4.4-fold increase of the Ezrin-pThr567 phosphorylation level at 72 h post-transfection while no or poor increase was observed at 24 h and 48 h post-transfection, respectively, relative to its level in mock-transfected cells. Values were normalized to the amount of total Ezrin in each sample.

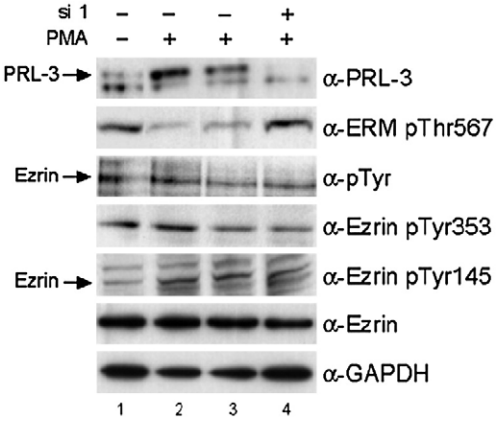

Fig. 4. Increased expression of PRL-3 in HUVEC upon PMA treatment produced dephosphorylation of Ezrin-pThr567. Confluent HUVEC were treated for 24 h with DMSO/PMA 100 nM (lane 2) or mock-treated with DMSO alone (lane 1). For reversion of PRL-3 induction, HUVEC pre-incubated with PMA were transfected with the PRL-3 specific silencing RNA si1 (10 nM) (lane 4) or mock-transfected (lane 3) and further incubated in the presence of PMA for 24 h. Total protein extracts were resolved on 7.5% SDS-PAGE and transferred to nitrocellulose membranes for evaluating the amount of PRL-3, total Ezrin and its phosphorylated forms. GAPDH was used as a reference protein. The bands corresponding to PRL-3 and Ezrin are indicated by arrows.

3.3. PRL-3 knock-down in HCT116 enhances phosphorylation of Ezrin-Thr567

Down-regulation of the endogenous PRL-3 mRNA in HCT116 cells was achieved by RNA interference in order to confirm Ezrin as a specific cellular target of PRL-3. Transfection of HCT116 cells with PRL-3-directed siRNA oligonucleotides produced a significant reduction of the PRL-3 mRNA. Only about 25% of the endogenous PRL-3 mRNA was present 24 h post-transfection, while this amount increased to 35% at 72 h post-transfection, as measured by Real Time PCR (Fig. 3A). The reduction of PRL-3 mRNA 24 h post-transfection produced the almost complete disappearance of the PRL-3 protein, as judged by western blot using a polyclonal antibody directed against the PRL-3 protein expressed in *E. coli*, which also recognized unspecific bands (Fig. 3B). This low level was maintained up to 72 h post-transfection, despite the increase of the mRNA level (Fig. 3B, lane 3).

Variations in the overall phospho-tyrosine level and in the phosphorylation state of Ezrin-Thr567 were monitored by western blot analysis of total extracts from cells transfected with the siRNA (Fig. 3C, lanes 4 to 6) in comparison to those from mock-transfected cells (Fig. 3C, lanes 1 to 3). As shown in Fig. 3C, PRL-3 down-regulation did not appear to affect the overall tyrosine-phosphorylation level of Ezrin. In contrast, an increased level of phosphorylation at Ezrin-Thr567 was observed in cells transfected with siRNA 72 h post-transfection (Fig. 3C, lane 6). The observation that higher levels of Ezrin-pThr567 were only observed at 72 h post-transfection might imply that Ezrin-pThr567 has to accumulate to be detectable by western blot. These results confirmed Ezrin as a specific PRL-3 target and pointed to Thr567, one of the most critical residues for regulation of Ezrin functions, as the main residue hit by PRL-3 action. On the other hand, the unaltered levels of Ezrin-tyrosine phosphorylation might either suggest that the effect observed upon ectopic PRL-3 over-expression was unspecific or that only high levels of PRL-3 expression, such as those possibly reached in metastatic cells, are needed to affect pTyr levels.

3.4. PRL-3 affects only Ezrin-pThr567 in endothelial cells

A significant increase of PRL-3 expression in endothelial cells upon PMA treatment has been recently reported [27] though the PRL-3 expression level hardly reaches that obtained in HCT116 cells by ectopic over-expression. Therefore, we sought to verify whether the induction of the PRL-3 endogenous gene might reproduce the same effects on the Ezrin phosphorylation status observed by ectopic over-expression in HCT116 cells. We decided to use HUVEC (Human Umbilical Vascular Endothelial Cells) which express a very low PRL-3 level in standard condition but that showed a significant increase of PRL-3 expression upon PMA treatment (100 nM) (Fig. 4, α-PRL-3, compare lanes 1and 2). Notably, neither variation of the phosphorylation status of Ezrin-pTyr145 and Ezrin-pTyr353 nor that of the Ezrin global tyrosine-phosphorylation status was observed by probing cell extracts with the specific antibodies (Fig. 4, lanes 1 and 2). The only Ezrin residue whose phosphorylation level appeared affected by the increased expression

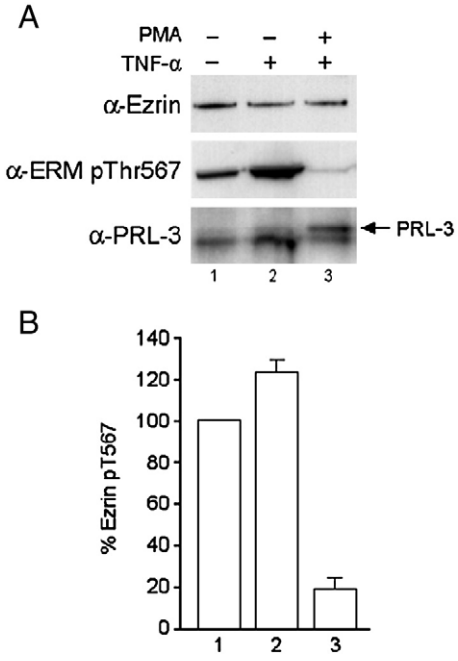

Fig. 5. PMA treatment of HUVEC cells abrogates the effect of TNF-α in enhancing phosphorylation of Ezrin-Thr567. A) HUVEC that were (lane 3) or were not (lanes 1 and 2) treated with PMA/DMSO were further incubated with 20 ng/ml TNF-α (lanes 2 and 3) or vehicles alone (PBS/0.1% BSA and DMSO) (lane 1). Total Ezrin, Ezrin-pThr567 and PRL-3 were examined by western blot. B) TNF-α treatment caused 30% increase of the phosphorylation level of Ezrin-Thr567, while the enhanced expression of PRL-3 in cells simultaneously treated with TNF-α and PMA caused 80% reduction with respect to its degree of phosphorylation in mock-treated cells set to 100%, as derived from quantification of the corresponding bands in the immunoblots. Values were normalized to the amount of total Ezrin in each sample.

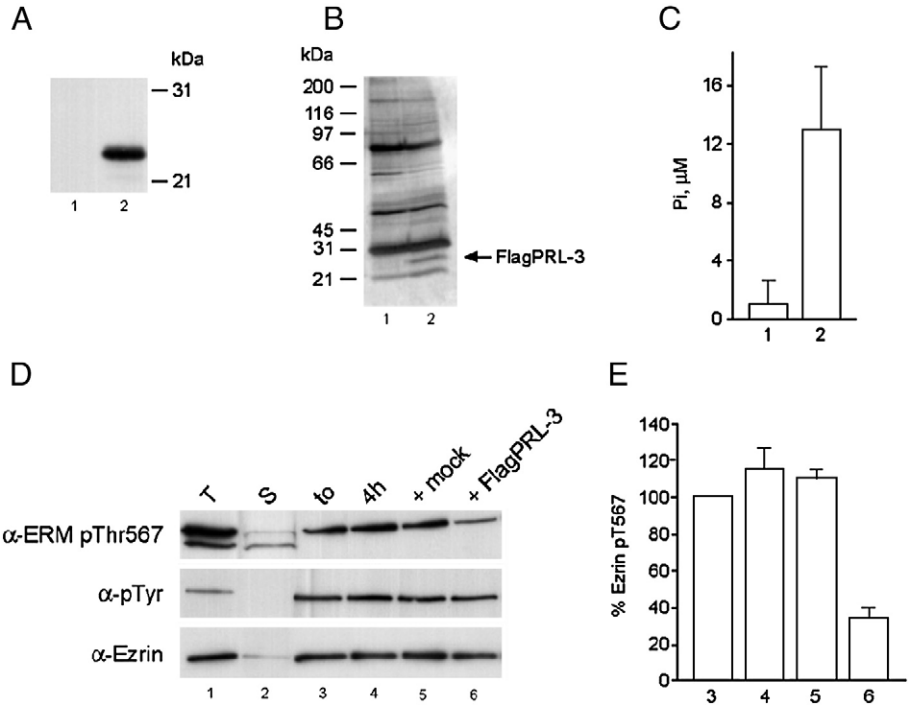

Fig. 6. Ezrin-pThr567 is a substrate of the PRL-3 enzyme. For *in vitro* PTPase assays using PRL-3 as enzyme and phosphorylated Ezrin as substrate, a FlagPRL-3 protein was immunoprecipitated from transiently transfected HCT116 cells with Anti-Flag M2 resin followed by native elution competing with a Flag peptide. As a control, mock-transfected cells were subjected to the same procedure. A) The presence of PRL-3 in native total extracts from FlagPRL-3- (lane 2) and mock- (lane 1) transfected cells was assessed by immunoblotting and B) the total protein pattern of mock (lane 1) and FlagPRL-3 (lane 2) extracts eluted from the anti-Flag M2 resin were compared by silver-staining of SDS-gels. C) *In vitro* phosphatase assays using DiFMUP as substrate confirmed the presence of phosphatase activity especially in the FlagPRL-3 eluted sample (bar 2) with respect to mock (bar 1). Phosphatase activity is expressed as the concentration of free phosphate released upon 4 h incubation at 37 °C. D) Endogenous phospho-Ezrin was immunoprecipitated from total extracts (T, lane 1) of HCT116 cells with anti-Phospho-tyrosine (4G10) agarose-conjugate and a negligible amount still remained in the unbound material (S, lane 2). The agarose-bound phospho-Ezrin was either not incubated (to, lane 3) or incubated for 4 h at 37 °C either in reaction buffer alone (lane 4) or with mock (lane 5) or FlagPRL-3 (lane 6) immunoprecipitated samples. The level of global Ezrin-pTyr and pThr567 in each sample was analyzed by western blot, using total Ezrin as internal control. E) Incubation with FlagPRL-3 produced 70% reduction of phosphorylation of Ezrin-Thr567 (bar 6) with respect to its level in the initial material (bar 3) set to 100%, as judged by quantification of immunoblots using total Ezrin as internal reference. Bars numbers correspond to lanes numbers in panel D.

of PRL-3 was Thr567 (Fig. 4, α-ERMpThr567). In order to exclude the possibility that the effect observed might result from PMA action other than PRL-3 up-regulation, we transfected cells with the PRL-3-directed siRNA si1 following PMA treatment. As shown in Fig. 4 (α-PRL-3, lanes 3–4), transfection of the silencing RNA completely counteracted PMA action re-establishing the PRL-3 expression level of naïve HUVEC (lane 1). The effect on the phosphorylation status of Ezrin-Thr567 also appeared abolished being the level of pThr567 in the silenced cells (Fig. 4, α-ERMpThr567, lane 4) comparable to that of untreated HUVEC (lane 1). PRL-3 mRNA silencing in the PMA-treated cells did not alter the tyrosine-phosphorylation state of Ezrin (Fig. 4, lanes 1, 3, 4).

Additional experiments were performed to verify whether PMA induction of PRL-3 expression in endothelial cells could counteract the known effect of TNF-α in enhancing ERM-Thr567 [28]. While TNF-α treatment of HUVEC caused an increase in the level of Ezrin-pThr567 (Fig. 5A, compare lanes 1 and 2), the enhanced expression of PRL-3 in cells simultaneously treated with TNF-α and PMA caused a great reduction of Ezrin-pThr567 level (Fig. 5A, lane 3), which was estimated as about 20% of that present in untreated HUVEC (Fig. 5B). These data strongly indicate that PRL-3 is involved in the dephosphorylation of Ezrin-Thr567 in endothelial cells.

3.5. Phosphorylation of Thr567 is decreased by PRL-3 in vitro

The experiments described above clearly suggested Ezrin, and particularly Ezrin-pThr567, as a specific cellular target of the PRL-3 phosphatase. However, there were no indication of whether the effects observed derived from a direct action of PRL-3 on Ezrin or, on the contrary, result from its activity toward a second phosphatase (or other effectors) that, in turn, affects Ezrin phosphorylation status. In order to clarify this point, we devised an experiment aimed to measure *in vitro* the phosphatase activity of PRL-3 extracted from cells using immunoprecipitated Ezrin as a substrate. To this end, an N-terminally flagged PRL-3 protein (FlagPRL-3) was over-expressed in HCT116 cells by transient transfection (Fig. 6A) and partially purified 24 h after transfection by immunoprecipitation with an α-Flag antibody followed by elution from the α-Flag-resin with high excess of a Flag peptide. As a control, mock-transfected cells were treated in the same way of those over-expressing FlagPRL-3 and the pattern of the proteins eluted from mock and FlagPRL-3 containing

extracts compared by silver-staining of SDS-gels. As shown in Fig. 6B, the proteins eluted from the α-Flag-resin were essentially the same in both the extracts with FlagPRL-3 as the only band, among the many others, that was clearly absent in the partially purified material from mock-transfected cells. To exclude the possibility that phosphatases other than FlagPRL-3 could be present among the contaminating proteins, the phosphatase activity of partially purified extracts from mock and FlagPRL-3 transfected cells was measured on the generic phosphatase substrate DiFMUP. The experiment reported in Fig. 6C demonstrates that a significant phosphatase activity was contained in the FlagPRL-3 preparation but was almost absent in the control extract. Ectopic over-expression in HCT116 of FlagPRL-3 still produced reduction of both Ezrin tyrosines and Thr567 phosphorylation levels (not shown), thus excluding a possible negative effect of the Flag peptide.

Because of the low immunoprecipitation efficiencies of all the commercial α-Ezrin antibodies tested, we decided to use an agarose-conjugated α-pTyr antibody to enrich Ezrin from HCT116 cells. To preserve the complete phosphorylation status of Ezrin on Tyr and Thr residues, cells were treated with H_2O_2/orthoVanadate and extracts prepared in the presence of common phosphatases inhibitors. As shown in Fig. 6D (lanes 1, 2, 3), Ezrin was efficiently enriched on the α-pTyr/agarose beads (Fig. 6D, α-Ezrin) and, in the conditions adopted, the phosphorylation status of both Tyrosine residues and Ezrin-Thr567 was preserved (Fig. 6D, α-pTyr and α-ERM-pThr567). Following extensive washing to get rid of the phosphatase inhibitors present in the cell extracts, the Ezrin/α-pTyr beads were incubated for 4 h at 37 °C either alone or in the presence of partially purified FlagPRL-3 or control preparation. Interestingly, while no effect was observed on the global tyrosine-phosphorylation status of Ezrin (Fig. 6D, lanes 4,5,6, α-pTyr), a remarkable reduction of the phosphorylation level associated to Ezrin-Thr567 was obtained upon incubation with FlagPRL-3 (Fig. 6D, lanes 4,5,6, α-ERMpThr567) which was estimated as corresponding to about 30% of that of the untreated or mock-treated samples (Fig. 6E). These results might indicate Ezrin-pThr567 as a specific and direct target of PRL-3.

4. Discussion

In this study we present evidence pointing to Ezrin as a specific and direct cellular target of PRL-3. With the aim of identifying its substrate(s), we decided to use HCT116 cells since PRL-3 expression level has been observed as being particularly enhanced in colon cancer metastasis and HCT116 were thought to contain the PRL-3 cellular target(s) being a colon cancer cell line with metastatic potential. In addition, as the expression level of the endogenous PRL-3 protein in HCT116 is limited if compared to other colon cancer cell lines ([27] and data not shown), we thought that ectopic over-expression of PRL-3 in this cell line might have produced a notable modification of its substrate(s) thus allowing its identification. Indeed, Ezrin was identified in HCT116 among the cellular proteins whose phosphorylation level resulted affected by ectopic over-expression of wtPRL-3, but not of its catalytically inactive mutants. That Ezrin is in fact the major target of PRL-3 in HCT116 has been also demonstrated by 2-D DIGE (data not shown), which has been described as a powerful technique for monitoring changes in the protein phosphorylation pattern. In this case, however, less pronounced alterations of the phosphorylation pattern or expression level of other proteins were also observed. The results of the 2-D DIGE experiments will be described in deep detail elsewhere.

The identification of Ezrin as the predominant target of PRL-3 action in HCT116 cells is of particular interest because of the involvement of the ERM family members (Ezrin-Radixin-Moesin) in a number of cellular processes, such as cell survival, proliferation, invasiveness and migration, where PRL-3 also takes part [23]. Moreover, Ezrin expression correlates with tumor thickness and level of invasion and it might play a role in the metastatic progression of several tumors [29,30]. The biological role of Ezrin in the cascade of tumor growth and metastasis is, however, not yet completely understood but it is known that Ezrin resides at the nexus of several pathways which regulate cellular behavior influencing metastatic potential [29]. Furthermore, its action is controlled by the phosphorylation status of several Tyr, Ser and Thr residues and while the many kinases involved are well studied, little is known about the phosphatases implicated [31–33].

In HCT116 cells, Ezrin is one of the most abundant and heavily phosphorylated proteins, especially at the level of tyrosine residues. For this reason, the finding that PRL-3 over-expression caused a heavy dephosphorylation of pTyr-Ezrin and, as a consequence, also of the two specific tyrosines analyzed, Tyr353 and Tyr145, might well suggest an unspecific action of PRL-3 on Ezrin tyrosine-phosphorylation levels. Moreover, the observation that suppression of endogenous PRL-3 in HCT116 cells by RNA interference did not appear to affect the tyrosine-phosphorylation state of Ezrin, seems to exclude a role of PRL-3 in the balance of Ezrin tyrosine-phosphorylation pattern. However, the possibility exists that Ezrin-tyrosine residues might only be affected by high expression levels of PRL-3, such as those possibly reached in cancer metastasis, while lower PRL-3 expression does not.

As well as the other ERM proteins, Ezrin exists in the cytoplasm in an inactive, “closed”, conformation with N-terminal to C-terminal association within the protein or with other ERM members [22]. Upon threonine and tyrosine phosphorylation, Ezrin assumes an active, “open”, conformation, and bridges F-actin directly or indirectly to the cell membrane [22]. Thr567 plays a major role in Ezrin activity since its phosphorylation is the crucial part of the process that triggers the active “open” conformation of the molecule [26]. Notably, as a further consequence of wtPRL-3 ectopic over-expression, the degree of Ezrin-Thr567 phosphorylation also appeared greatly diminished. That the PRL-3 effect might be specific was suggested by the observation that the phosphorylation state of the corresponding Thr residue in Radixin did not appear altered by PRL-3 over-expression, though contained in a sequence that is well conserved among the ERM proteins [31]. Indeed, the RNA interference experiments, where an enhancement of Ezrin-pThr567 abundance was observed upon endogenous PRL-3

suppression, confirm a PRL-3 action on Ezrin-pThr567. In addition, the *in vitro* PTPase experiments, where immunoprecipitated Ezrin was subjected to dephosphorylation by an immunoprecipitated PRL-3 carrying an N-terminal Flag epitope (FlagPRL-3), seem to suggest Ezrin-pThr567 as a direct substrate of PRL-3, though the possibility cannot be excluded that, in these experiments, the PRL-3 action on Ezrin might have been mediated by other phosphatase(s), direct target(s) of PRL-3, co-immunoprecipitated by the α-pTyr antibody used to enrich Ezrin from the cell extracts. Intriguingly, however, Ezrin-pTyr residues were not affected. Taken together, these results point to Ezrin-pThr567 as a specific cellular target of the PRL-3 phosphatase activity. Little, if anything, is known about the substrate preferences of PRL-3 or its close homologues PRL-1 and 2, though they can be classified by structure-based sequence alignments as dual specificity PTPases [34] and the results of our *in vitro* PTPase assays might support this classification. The observation that in the *in vitro* PTPase assays we noted PRL-3 action only on Ezrin-pThr567 does not exclude the possibility that, on other proteins, PRL-3 might act on pTyr residues. On the other hand, besides not being direct substrates of PRL-3, our failure to observe direct dephosphorylation of Ezrin-pTyr residues might also have additional explanations: i) the α-pTyr antibody used to enrich Ezrin from the cell extracts might have impeded recognition of the pTyr residues, in contrast with our assumption that statistically, most, if not all, of them could have been accessible to the enzyme; ii) in the hypothesis that very high levels of PRL-3 are needed for Ezrin-pTyr dephosphorylation to occur, as suggested above, the enzyme might have a very low affinity toward Ezrin-pTyr residues, such that the possibly low amount of active protein in the immunoprecipitated material is not sufficient to catalyze dephosphorylation; iii) accessory factor(s) or previous modification of the target protein are also required but are not present in the *in vitro* reaction with the consequence that the *in vitro* assay does not recapitulate the in cell process.

Besides reinforcing the suggestion of Ezrin-pThr567 as a target of PRL-3, the observation that enhancement of expression of endogenous PRL-3 by PMA stimulation of HUVEC produces dephosphorylation of Ezrin-pThr567 is of particular interest because of the possible role played by PRL-3 in triggering angiogenesis and establishing the microvasculature needed for tumor survival and progression [16,18,27]. In endothelial cells, phosphorylation of Ezrin-Thr567 plays a central role in promoting the cellular responses to TNF-α treatment, including inhibition of proliferation and impaired ability to progress through the cell cycle [28,35]. In this light, dephosphorylation of Ezrin-Thr567 guided by PRL-3 might likely have an opposite effect by enhancing cell proliferation and therefore promoting EC proliferation and angiogenesis. Indeed, PMA stimulation of ECs has been shown to enhance invasion, tube formation and proliferation and PRL-3 was reported among the 10 genes with the largest increase of expression on PMA treatment [27]. In addition, PRL-3 was found in the endothelial cells of tumor vasculature, regardless of tumor source [8] and as one of the genes predominately expressed in the vasculature of invasive breast cancer [18]. It is noteworthy that in PMA-stimulated HUVEC PRL-3 only affected phosphorylation of Ezrin-pThr567 with no appreciable effect on the tyrosine-phosphorylation levels. In HUVEC, the PRL-3 level upon PMA induction is still well below that reached by ectopic over-expression, thus strengthening the suggestion that only very high PRL-3 levels might affect tyrosines phosphorylation.

On the assumption that aberrant PRL-3 expression in metastatic cells does affect both global Ezrin-tyrosine phosphorylation and Thr567, the heavy dephosphorylation produced would likely culminate in Ezrin inactivation. Interestingly, recent studies report increased RhoA, RhoC and Src activities in cells expressing exogenous PRL-3, as well as decreased Rac1 [19,20]. Both RHO-family proteins and Src kinase are fundamental in controlling Ezrin function [25,36,37] and deregulation of their activity can promote tumor progression and metastasis [38,39] by altering actin cytoskeleton organization. Notably, small GTPase Rho controls ERM function by phosphorylating the C-terminal Thr, Thr567 in the case of Ezrin, required for ERM to adopt an active conformation. In turn, active ERMs control small GTPase Rho activity by recruiting both their positive (GEFs) and negative (RhoGDI) regulators [40]. It is conceivable that inactivation of Ezrin by PRL-3 action would promote release of Rho regulators thus explaining the increment of RhoA and RhoC activities, as well as the decreased Rac1, observed by Fiordalisi et al. [19]. Tyrosine kinase Src directly promotes phosphorylation of Tyr477 and Tyr145 in active Ezrin and the latter modification is linked to signaling events leading to epithelial cell spreading and proliferation [25,37]. However, for Tyr145 phosphorylation by Src to occur, Ezrin must be in an open, active conformation and binding of the Src-SH2 domain to phosphorylated Ezrin-Tyr190 is an additional fundamental prerequisite. It is likely, that in cells where PRL-3 is abnormally expressed, because of Ezrin inactivation, Src activity moves towards other targets initiating signal pathways involving ERK1/2, STAT3 and $P130^{CAS}$ which contribute to the increased cell invasion and migration properties found in cells over-expressing PRL-3 [20]. Therefore, loss of active Ezrin prompted by PRL-3 might contribute to tumor progression and development by deregulating RHO GTPase and Src activities.

We have provided evidence that Ezrin is a specific and direct cellular target of PRL-3. Although PRL-3 over-expression in the HCT116 colon cancer cell line appeared to affect Ezrin phosphorylation status at both tyrosine residues and Thr567, silencing experiments, combined to *in vitro* dephosphorylation assays, suggested that PRL-3 more directly acts on Ezrin-Thr567 indicating this enzyme as one of the phosphatases that function as modulators of Ezrin activity through regulation of the phosphorylation state of this crucial residue. Furthermore, the same effect on pThr567, but not on pTyr residues, was observed in endothelial cells confirming pThr567 as a specific substrate of PRL-3. We propose dephosphorylation of Ezrin-pThr567 by PRL-3 as a mean through which this phosphatase exerts its function in supporting new vasculature formation for tumor survival and progression, though the understanding of the precise molecular mechanism warrants further investigation. Moreover, we suspect that the aberrant expression of PRL-3 in metastatic cells will produce heavy dephosphorylation of Ezrin which might ultimately lead to Ezrin inactivation and promotion of the molecular processes at the basis of metastasis progression.

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