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EXPERIMENTAL CELL RESEARCH 315 (2009) 2888–2898

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Research Article

The PDZ protein MPP2 interacts with c-Src in epithelial cells

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ARTICLE INFORMATION

Article Chronology:
Received 7 August 2008
Revised version received 21 July 2009
Accepted 31 July 2009
Available online 6 August 2009

Keywords:
PDZ
Dlg
MAGUK
Src
Breast cancer cells
Cytoskeleton
Phosphorylation

ABSTRACT

c-Src is a non-receptor tyrosine kinase involved in regulating cell proliferation, cell migration and cell invasion and is tightly controlled by reversible phosphorylation on regulatory sites and through protein–protein interactions. The interaction of c-Src with PDZ proteins was recently identified as novel mechanism to restrict c-Src function. The objective of this study was to identify and characterise PDZ proteins that interact with c-Src to control its activity. By PDZ domain array screen, we identified the interaction of c-Src with the PDZ protein Membrane Protein Palmitoylated 2 (MPP2), a member of the Membrane-Associated Guanylate Kinase (MAGUK) family, to which also the Discs large (Dlg) tumour suppressor protein belongs. The function of MPP2 has not been established and the functional significance of the MPP2 c-Src interaction is not known. We found that in non-transformed breast epithelial MCF-10A cells, endogenous MPP2 associated with the cytoskeleton in filamentous structures, which partially co-localised with microtubules and c-Src. MPP2 and c-Src interacted in cells, where c-Src kinase activity promoted increased interaction of c-Src with MPP2. We furthermore found that MPP2 was able to negatively regulate c-Src kinase activity in cells, suggesting that the functional significance of the MPP2-c-Src interaction is to restrict Src activity. Consequently, the c-Src-dependent disorganisation of the cortical actin cytoskeleton of epithelial cells expressing c-Src was suppressed by MPP2. In conclusion we demonstrate here that MPP2 interacts with c-Src in cells to control c-Src activity and morphological function.

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Abbreviations: Dlg, Discs large; L27, Lin-2/Lin-7 domain; FERM, (four point 1, ezrin, radxin, moesin); IF, Immunofluorescence; MAGUK, Membrane-Associated Guanylate Kinase; MPP2, Membrane Protein Palmitoylated 2; PDZ, Postsynaptic density-95/Discs large 1/Zonula Occludens-1; SH, Src homology; Wb, Western blot

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doi:10.1016/j.yexcr.2009.07.028

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Introduction

c-Src regulates a wide range of cellular functions including cell proliferation and cell migration [1,2]. Proper regulation of Src kinases is important and the C-terminal deletion in the viral oncogene v-Src of the Rous Sarcoma Virus leads to deregulated Src activity and oncogenic transformation [1,2]. Deregulation of c-Src is believed to play an important role in human tumourigenesis by promoting proliferation, survival and migration of tumour cells [3]. The regulation of c-Src is complex and involves the reversible phosphorylation of Y416 and Y527 as well as protein–protein interactions through its SH2 and SH3 domains [4]. Elimination of the PSD95/Dlg/ZO-1 (PDZ) ligand sequence in c-Src increases the potential of c-Src to promote an invasive phenotype in epithelial cells [5]. With the adherens junction PDZ protein AF-6 one candidate protein has been identified to inhibit c-Src function [6], but additional PDZ proteins are likely implicated in regulating c-Src function.

Scaffolding proteins of the Membrane-Associated Guanylate Kinase (MAGUK) family tether adhesion molecules, receptors and components of intracellular signalling pathways to spatially organise multi-component signalling complexes [7]. MAGUKs contain a catalytically inactive guanylate kinase (GuK) domain, a Src-homology 3 (SH3) domain and one or several PSD95/Dlg/ZO-1 (PDZ) domains. Membrane Protein Palmitoylated 2 (MPP2) has previously been isolated in a screen for cDNAs subjected to allelic losses on chromosome 17q12–q21, a genomic region frequently altered in breast cancer cells [8]. MPP2 has two L27 domains at its N-terminus in addition to the conserved PDZ-SH3-GuK module. MPP2 is structurally similar to Discs large (Dlg), one of the founding and well-characterised members of the MAGUK family [9]. MAGUK proteins including Dlg are considered to act as tumour suppressor proteins in vertebrates because of their essential structural and regulatory role in tissue organisation and maintenance and in cell signalling.

Here we identified the interaction between the MAGUK protein MPP2 and c-Src. Localisation and function of MPP2 in cells are unknown. We investigated the function of MPP2 and its interaction with c-Src. We characterised the localisation of endogenous MPP2 in human breast epithelial cells and of overexpressed MPP2 in HEK293 cells. We detected MPP2 in the cytoskeleton, where it partially co-localised with microtubules and c-Src. The c-Src kinase activity allowed MPP2 phosphorylation and increased the interaction between MPP2 and c-Src. Conversely, MPP2 negatively regulated c-Src kinase activity and prevented disorganisation of the actin cytoskeleton in lamellipodia of cells overexpressing c-Src. Our data thus indicate that MPP2 and c-Src constitute a protein complex to restrict c-Src activity and function in cells.

Materials and methods

Cells

The MCF-10A cells were obtained from the American Type Culture Collection and grown in complete growth medium (DMEM/F12 (Gibco) with 2 mM L-glutamine supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma), 100 ng/ml cholera toxin (Sigma), 10 ng/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma) and 5% horse serum). Starvation medium is DME-F12 without supplements. HEK293 and COS-7 cells were grown in DME medium (Gibco) containing 10% foetal calf serum (FCS). Starvation medium was DME medium without FCS. Chicken c-Src amino acid numbering is used throughout this article.

Constructs and expression vectors

Human MPP2 isoform 2 was obtained as cDNA clone (RZPD ID: IRAKp961N0662Q2, Germany) and subcloned into pFLAG-CMV-2 (SIGMA). Truncated MPP2 constructs were generated by PCR and subcloned into the EcoRI and SalI sites in pFLAG-CMV-2. Single point mutations were introduced by site directed mutagenesis (Stratagene). The TAT codon coding for Y was replaced with GCT (Ala) or GAT (Asp), respectively and the sequences were verified by sequencing. HA-tagged c-Src constructs are described elsewhere [5,6]. For all overexpression experiments, FLAG-tagged MPP2 and HA-tagged Src proteins were used.

PDZ array

TranSignal PDZ arrays I–IV (Panomics) were screened for interaction with Src by incubating the arrays with cell extracts containing overexpressed HA-c-Src as described recently [10].

Cell transfection

HEK293 and COS-7 cells were transfected using JetPEI (Polyplus) according to the manufacturer's instructions. 24 h after transfection, growth medium containing 10% FCS was replaced with fresh growth medium or with starvation medium without FCS. 48 h after transfection, cells were lysed and lysates were processed for immunoprecipitation analysis. Alternatively, cells were fractionated as described below. Where indicated, EGF was used at a concentration of 50 ng/ml.

Cell lysis, Western blot (Wb) and antibodies

Cells were lysed in modified radio-immunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 50 mM Tris (pH7.4), 1 mM EDTA (pH 8.0), 1.0% NP40, 0.25% Na-Deoxycholate, 2 mM Na-Vanadate, 25 mM NaF and protease inhibitor cocktail (Roche). Wb was carried out using standard procedures. Cellular fractionations were performed either by using FractionPREP (BioVISION, http://www.biovision.com/) according to the manufacturer's instructions or by ultracentrifugation as described recently [5]. Antibodies used were the following: mouse mc anti-Src clone GD11 and anti-phospho-Y 4G10 (Upstate); anti-Na^+/K^+ ATPase (468.8) (Abcam); rabbit pc anti-Src2, anti-focal adhesion kinases C-20, anti-cortactin H-191 and anti-Ephrin-B1 C-18 (Santa Cruz); anti-phospho-Src family (pY416) (Cell Signaling Technology). MPP2 is a custom-made (Eurogentec) rabbit polyclonal antibody raised against a 15-mer peptide in the C-terminus of human MPP2 and was used at a concentration of 250 ng/ml in Wbs and 2.5 μg/ml for IF.

Immunofluorescence microscopy

Cells were fixed/permeabilised in 100% MetOH at −20 °C for 3 min, treated with 0.5% TX-100 for 5 min and blocked with 10% FCS in PBS. Primary antibodies were used at 1/200 dilution in 5% FCS in PBS. Secondary anti-mouse or anti-rabbit antibodies (Jackson Immuno Research) coupled to FITC (fluorescein

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isothiocyanate) or TRITC (tetramethylrhodamine isothiocyanate) were used for detection. Confocal image stacks were acquired on an inverted Leica SP5 microscope through a Leica 63× oil objective, recorded with Leica application software and analysed with Imaris5.3 software.

Sequence comparison and phylogenetic analysis

Full-length amino acid sequences were downloaded from Swiss-Prot and TrEMBL. Sequence alignments and dendrogram generation were performed on ClustalW and visualised by TreeView software. Phosphorylation sites were identified using NetPhos software. ClustalW, TreeView and NetPhos are publicly available from ExPASy proteomic server.

Results

c-Src interacts with the PDZ domain of MPP2

We screened PDZ domain arrays for c-Src interaction [10] and identified the PDZ domain of Membrane Protein Palmitoylated 2 (MPP2) (Fig. 1A). MPP2 belongs to the Membrane-Associated Guanylate Kinase (MAGUK) family of proteins and consists of two

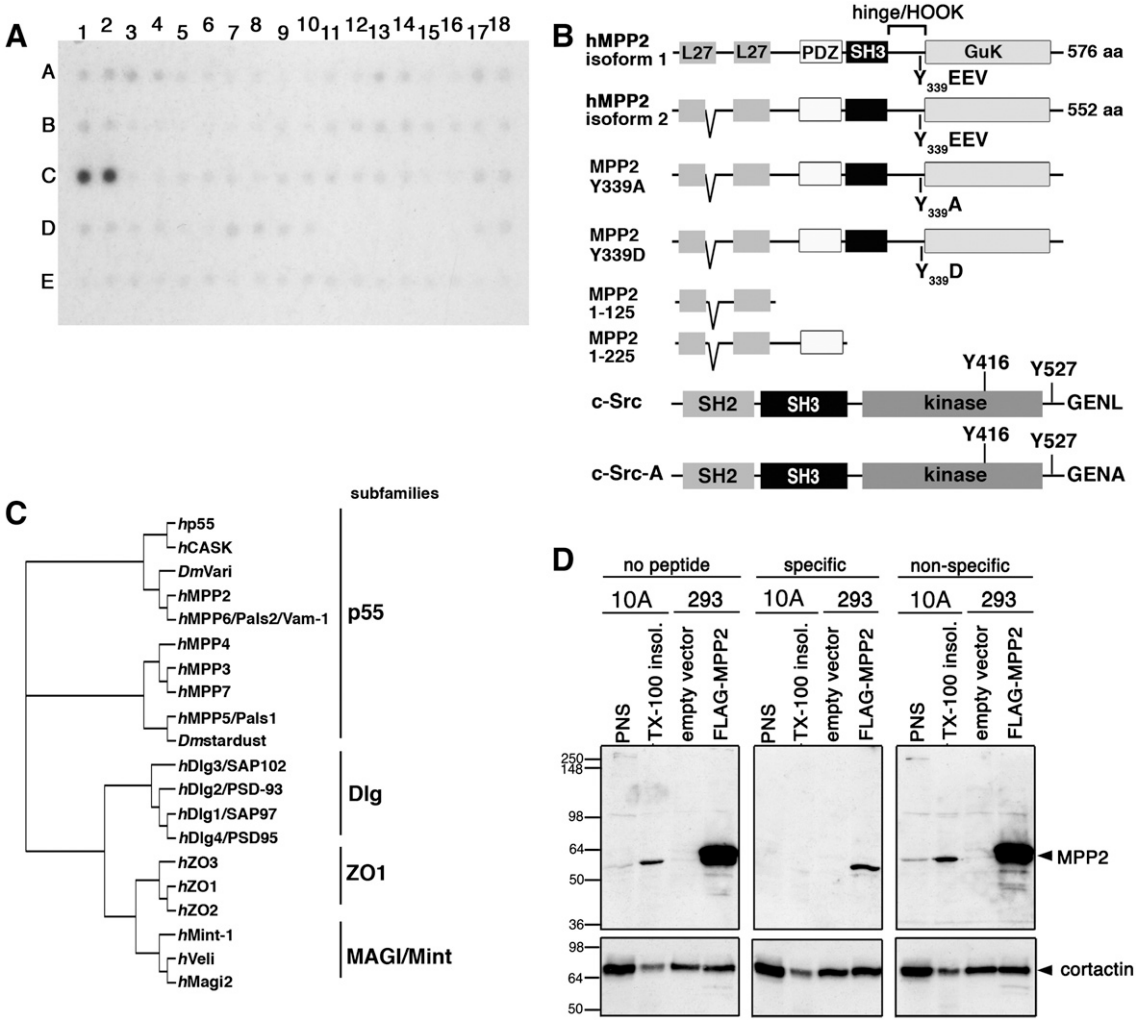

Fig. 1 – Interaction of c-Src with the MAGUK family PDZ protein MPP2. (A) PDZ array containing PDZ domains fused to GST and immobilised on membrane was analysed by Western blot (Wb) for HA-Src interaction. (B) Schematic view of human MPP2 isoforms 1 and 2 and mutated and truncated versions thereof used in this manuscript. All constructs used are N-terminally tagged with a FLAG epitope (not shown). Schematic view of c-Src with the C-terminal PDZ domain ligand sequence Gly-Glu-Asn-Leu (GENL) or the mutated version Gly-Glu-Asn-Ala (GENA [29]) shown. Y416 is phosphorylated in active c-Src and detected with anti-pY416Src antibody. Y527 is the negative regulatory tyrosine. (C) Dendrogram of human MAGUK family members and *D. melanogaster* Varicose (Vari) and stardust using full-length sequences. (Abbreviations: CASK: calcium/calmodulin-dependent serine protein kinase; Dlg: Discs large; MAGI: MAGUK with inverted orientation; Pals: Proteins-associated with Lin-2 and Lin-7; PSD: Postsynaptic Density; SAP: Synapses-associated protein; Vam: Veli-associated MAGUK; Vari: Varicose; ZO: Zonula Occludens). (D) Detection of endogenous MPP2 or overexpressed FLAG-MPP2 in MCF-10A (10A) or HEK293 (293) cell extracts, respectively. PNS is post-nuclear supernatant after detergent-free mechanical lysis and ultracentrifugation. TX-100 insol. is the TritonX-100-insoluble fraction after mechanical lysis and ultracentrifugation. Anti-MPP2 antibody was used without peptide (no peptide) or after pre-incubation with the antigen peptide (specific) or with a non-specific peptide (non-specific).

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L27 domains, a SH3 domain, a PDZ domain and a guanylate kinase (GuK) domain (Fig. 1B). In the present study, MPP2 isoform 2 was used, which is identical to isoform 1 except that it displays an alternatively spliced first L27 domain. To classify similarities between MAGUK family proteins, we generated a dendrogram based on full-length amino acid sequence homologies using ClustalW software (Fig. 1C). MPP2 groups with other MPPs and is most similar to MPP6/Pals2 [11]. MPP2 is closely related to *D. melanogaster* Varicose (Vari), which constitutes together with MPP2 and MPP6/Pals2 a new subgroup of basolateral MAGUKs [12]. However, the subcellular distribution, regulation and function of MPP2 are unknown. To investigate MPP2 function, we generated antibodies raised against a 15-mer peptide in the GuK domain. The affinity-purified antibody recognised the FLAG-tagged human MPP2 in Western blot analysis (Fig. 1D). The antibody also detected a protein of the expected molecular weight of approximately 60 kDa in the TX-100-insoluble fraction of the non-transformed human breast epithelial cell line MCF-10A. The signal from both the overexpressed FLAG-MPP2 and the endogenous MPP2 was specific because the immuno absorption was eliminated by competition with the antigen peptide (Fig. 1D). A non-specific peptide had no effect.

MPP2 localises to membranes and the cytoskeleton

To determine the subcellular distribution of MPP2 in MCF-10A cells, we fractionated the cells into cytosolic (c), membrane (m), nuclear (n) and cytoskeletal (cs) fractions. In cells grown in complete growth medium, MPP2 was mainly detectable in the cytoskeletal and weakly in the cytosolic fraction (Fig. 2A, left panel). The MPP2 antibody also detected higher molecular weight bands in the cytosolic fraction. In the membrane and the cytoskeletal fractions we also detected c-Src.

The MPP2 homologue Dlg1 functions as a tumour suppressor in flies [9]. The structurally related MPP2 could function analogously by contributing to the maintenance of the non-transformed state in epithelial cells. Therefore, we compared expression and subcellular localisation of MPP2 in MCF-10A cells to its expression and localisation in MCF-7 cells (Fig. 2A, right panels). MCF-7 is a transformed human breast cancer cell line with a weakly invasive phenotype [13]. In MCF-7 cells, we detected no MPP2 in the cytoskeletal fraction, whereas expression levels and distribution of c-Src, the focal adhesion kinase (FAK) and cortactin were similar to MCF-10A cells. Also in other established human breast cancer cell lines expression of MPP2 was reduced or not detectable (Supplementary Fig. S1). This suggested that MPP2 expression was impaired in breast epithelial cell lines that have undergone oncogenic transformation.

We next determined whether subcellular localisation of overexpressed FLAG-MPP2 in HEK293 cells was comparable to endogenous MPP2 in MCF-10A cells (Fig. 2B). We detected overexpressed FLAG-tagged MPP2 in all compartments, with comparable levels at the membrane and in the cytoskeleton. We then investigated whether the PDZ domain was sufficient to localise MPP2 to membranes, which could occur either by binding to a transmembrane ligand analogous to MPP6/Pals2 binding to the Nectin-like molecule (Necl-2) [14] or by direct interaction with the membrane [15]. We expressed the FLAG-tagged L27 domain without or with PDZ domain (MPP2 1–125

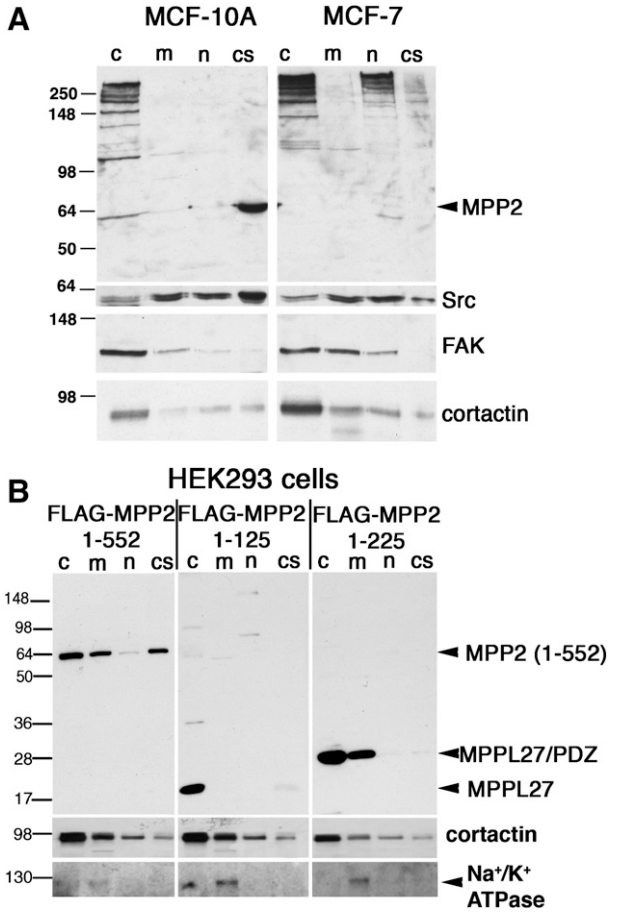

Fig. 2 – Interaction of MPP2 with membranes and the cytoskeleton. (A) MCF-10A and MCF-7 cells were fractionated into cytosolic (c), membrane (m), nuclear (n) and cytoskeletal (cs) fractions. Fractions were analysed by Wb with antibodies against the proteins indicated to the right of the panels. (B) HEK293 cells expressing FLAG-tagged versions of either MPP2 (MPP2 1–552), MPP2-L27 (MPP2 1–125) or MPP2-L27-PDZ (MPP2 1–225) were subjected to cell fractionation as described for A and analysed by Wb with anti-FLAG, anti-cortactin and anti-Na^+/K^+ antibodies.

or MPP2 1–225 respectively, see also Fig. 1B). Whereas FLAG-L27 remained cytosolic, FLAG-L27-PDZ was detectable in the membrane fraction, where we also detected the membrane marker sodium/potassium ATPase (Fig. 2B). Thus, MPP2 is likely localised to membranes by means of its PDZ domain; the SH3 or the GuK domain or both domains are required for MPP2 localisation to the cytoskeleton.

Filamentous, MPP2-positive structures co-localise with microtubules and c-Src

We next determined the localisation of endogenous MPP2 relative to microtubules and the nucleus in epithelial cells by confocal IF microscopy (Figs. 3A and B). MPP2 was visible in filamentous (arrows in Fig. 3A) and punctuated patterns (Fig. 3B). The filamentous patterns localised between nucleus and leading edge lamellipodium in the marginal cells of a migrating cell sheet (Figs. 3A and C), where they partially co-localised with microtubules

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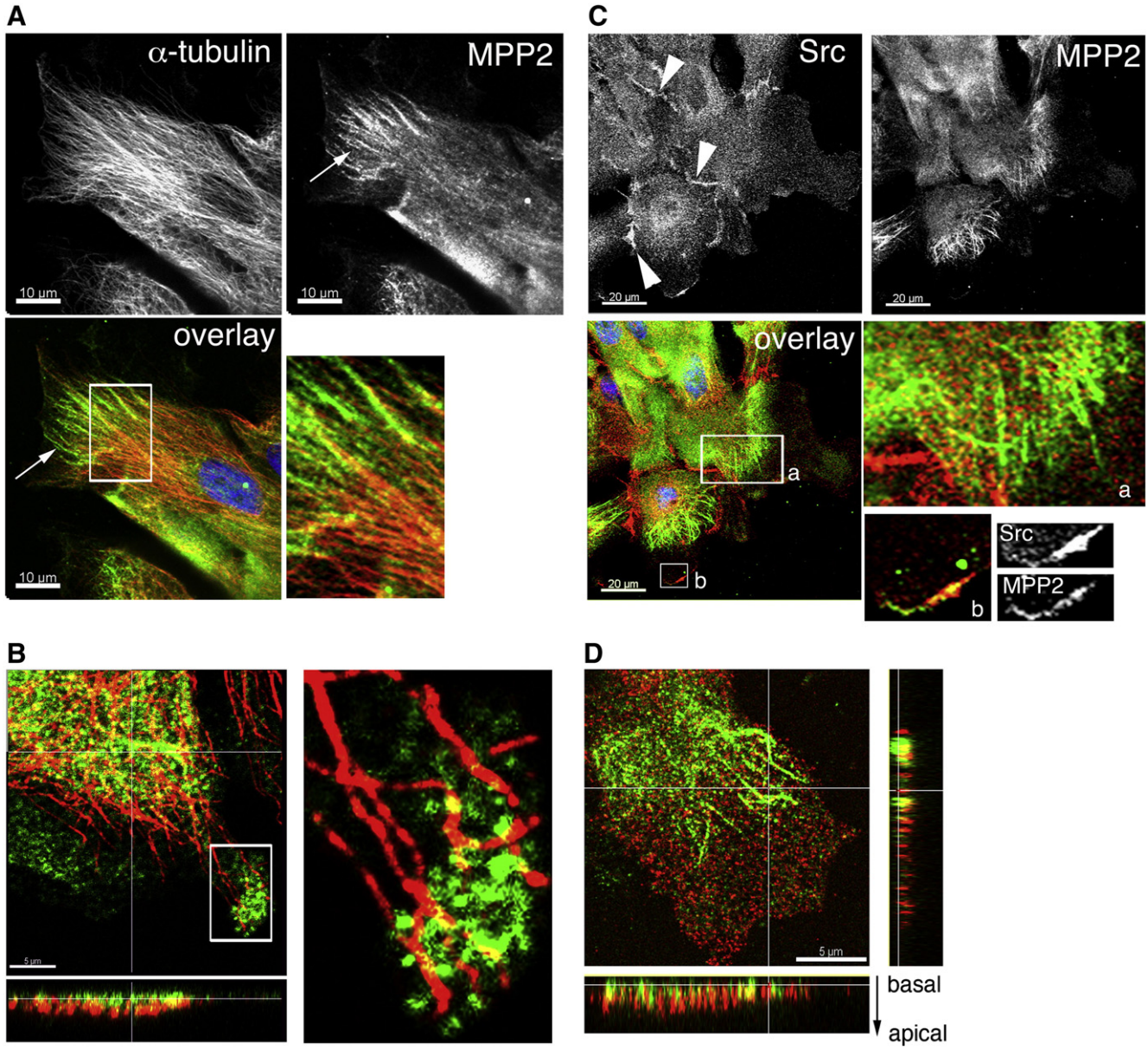

Fig. 3 – Co-localisation of filamentous MPP2 staining with microtubules and c-Src. (A) Confocal IF analysis of α-tubulin (red) and endogenous MPP2 (green) in MCF-10A cells grown in full growth medium. MPP2 is visible as filamentous structures (arrows) between nucleus and edge of lamellipodium. Co-localisation of microtubules and MPP2 are in yellow (magnified inset is 2.5-fold increase of framed area). Bars correspond to 10 µm. (B) EGF-stimulated MCF-10A cells (12 h after wounding and stimulation) were processed for confocal IF as described for panel A. Focus on basal side of lamellipodium (magnified inset is 4-fold increase of framed area). Bar corresponds to 5 µm. (C) Same treatment and image processing as described for panel A except that anti-Src antibody was used instead of anti-α-tubulin. Insets of higher resolution to the right of overlay are 4-fold magnifications of perinuclear region and of leading edge of lamellipodium in overlay. Bars correspond to 20 µm. (D) Same treatment as C. Basal cross sections along *X* and *Y* axes show co-localisation of c-Src and MPP2 at basal side of the cell. Bar corresponds to 5 µm.

(Fig. 3A, inset). We noted fewer and less marked filamentous MPP2 staining in confluent MCF-10A cells or in MCF-10A cells several lines behind the marginal cells during wound healing. Filamentous association of MPP2 depended on microtubules as it disappeared in the presence of nocodazole, an inhibitor of microtubule polymerisation (Supplementary Fig. S2). At the basal side of EGF-induced lamellipodia (Fig. 3B), MPP2 staining was punctuated with microtubules reaching into this region and partially co-localising with MPP2 (magnification).

We then determined the localisation of endogenous MPP2 relative to c-Src and the nucleus (Fig. 3C). We observed c-Src in perinuclear regions, in regions of cell–cell contact (arrowheads in Fig. 3C) and at the leading edge of lamellipodia. Filamentous MPP2 partially co-localised with perinuclear and basal c-Src (Figs. 3C,

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magnified in a, and D, cross sections) and at the leading edge of the lamellipodium (Fig. 3C, magnified in b). We observed no co-localisation of MPP2 and c-Src in regions of cell–cell contact (Fig. 3C). However, filamentous MPP2 co-localised with c-Src at the basal site of the cell (Fig. 3D). These data showed that in migrating MCF-10A cells, MPP2 is mainly localised to perinuclear regions and to filamentous structures between nucleus and leading edge, where it partially co-localises with microtubules and c-Src.

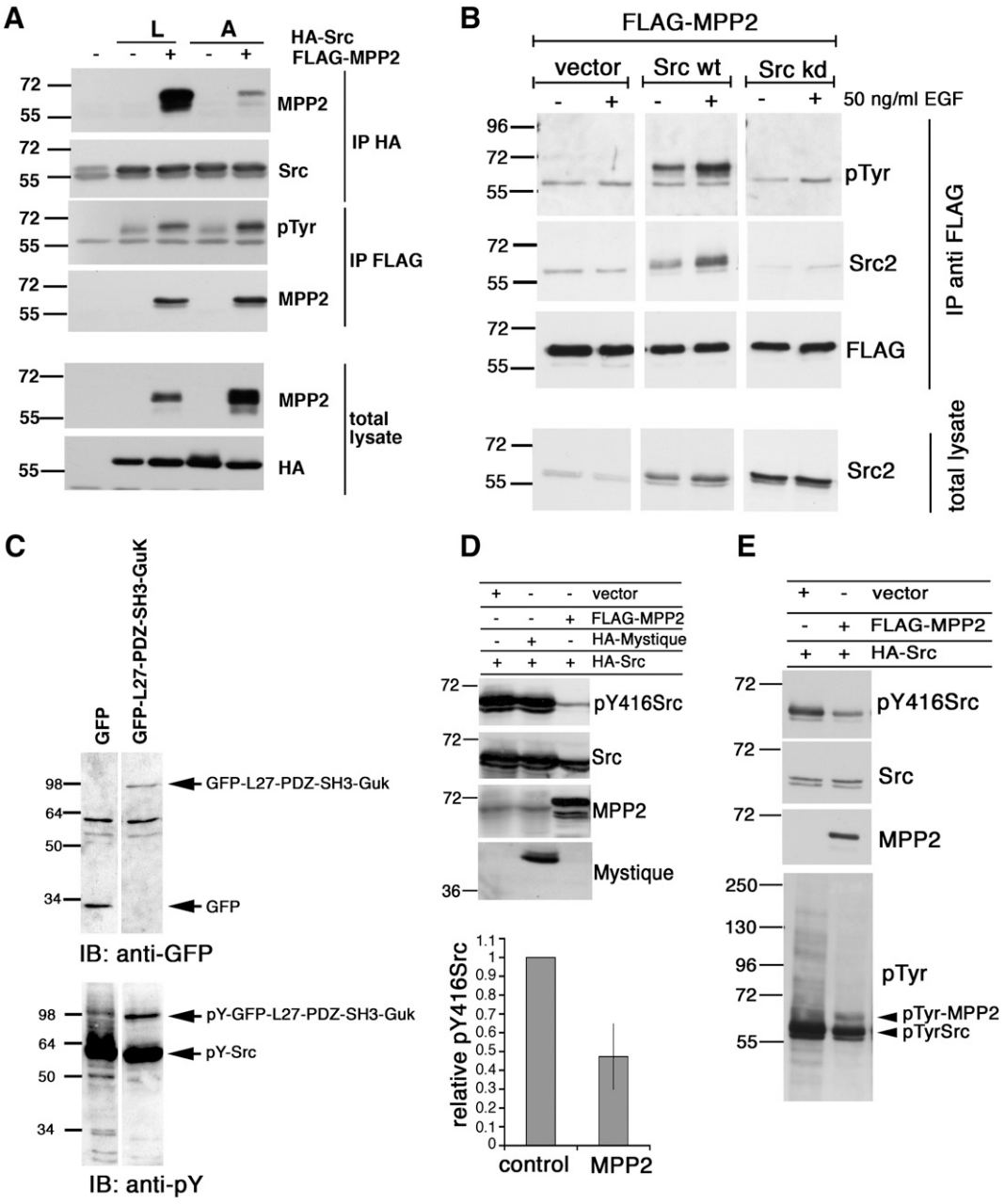

Fig. 4 – Src and MPP2 interact in cells. (A) Wild-type HA-Src-L or the PDZ ligand mutant HA-Src-A were co-expressed with FLAG-MPP2 in HEK293 cells. Anti-HA and anti-FLAG immunoprecipitates and total cell lysates from starved or EGF-stimulated cells were analysed by Wb using antibodies against the proteins indicated to the right of the panels. (B) HA-Src wt or kinase-deficient HA-Src kd were expressed together with FLAG-MPP2 in HEK293 cells. Anti-FLAG immunoprecipitates or total cell lysates from starved or EGF-stimulated cells were analysed by Wb using antibodies against the proteins indicated to the right of the panels. (C) Total cell lysates of HEK293 cells co-expressing HA-Src and GFP or the indicated GFP-MPP2 fusion proteins were analysed by Wb using anti-GFP (upper) or anti-pY (lower) antibodies. (D) HA-Src was co-expressed with control vector or FLAG-MPP2 or HA-Mystique. Total cell lysates were then probed for c-Src activity using anti-pY416Src and for total Src protein with anti-Src antibody. Intensities of bands from three independent experiments were quantified. The bar diagram shows c-Src activity relative to its protein expression with control HA-Mystique set at 100% and with MPP2 at 43% relative pY416Src. (E) MPP2 expression reduces overall protein tyrosine phosphorylation in cells. HA-Src was co-expressed with control vector or FLAG-MPP2. Total cell lysates were probed for c-Src activity using anti-pY416Src antibody and overall tyrosine phosphorylation with anti-pTyr antibody.

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Interaction of MPP2 and c-Src in cells

The PDZ domain of MPP2 bound c-Src *in vitro* (Fig. 1). We therefore tested whether the c-Src PDZ ligand sequence GENL in the c-Src C-terminus (Fig. 1B) is involved in the interaction between MPP2 and c-Src in cells. To this end, we compared association of MPP2 with the wild-type HA-Src (L) or HA-Src-A (A), a c-Src mutant where the C-terminal leucine in the PDZ ligand [6,10] was replaced with alanine (Fig. 4A). We expressed HA-Src or HA-Src-A alone or together with FLAG-MPP2 in HEK293 cells. HA immunoprecipitates were then analysed by Western blot. MPP2 co-precipitated with wild-type Src-L, but markedly less with Src-A, demonstrating that the PDZ ligand of c-Src contributes to the interaction of the two proteins. The fact that Src-A still precipitated MPP2, however, shows that additional binding sites exist. In FLAG immunoprecipitates we observed a Y-phosphorylated protein, which presumably is FLAG-MPP2 (see below). The intensities of these bands were independent of an intact PDZ-binding motif on c-Src.

In order to determine whether c-Src promoted tyrosine phosphorylation of MPP2, we co-expressed FLAG-MPP2 with HA-tagged wild-type Src (HA-Src wt) or the kinase-deficient mutant HA-Src kd in HEK293 cells. FLAG-MPP2 was immunoprecipitated from starved cells and cells stimulated with EGF. Only wild-type HA-Src, but not HA-Src kd was detected in the FLAG-MPP2 immunoprecipitates (Fig. 4B) confirming the interaction between Src and MPP2 and suggesting that Src kinase activity is required for this interaction. In HA-Src-expressing cells, but not in HA-Src-kd expressing cells, MPP2 was phosphorylated and phosphorylation of MPP2 increased upon stimulation with EGF. MPP2 phosphorylation was also inhibited when cells expressing HA-Src were treated with the Src kinase inhibitor PP2 (data not shown). Hence, Src kinase activity is required for MPP2 phosphorylation. In order to confirm the Src-dependent phosphorylation of MPP2 we also tested Y phosphorylation of MPP2 fused to GFP in HA-Src-expressing cells (Fig. 4C). The anti-pY antibody detected the GFP-L27-PDZ-SH3-GUK fusion protein, 98 kDa in size, but not GFP alone.

Since PDZ proteins are able to restrict c-Src function [16,17], we tested whether MPP2 could also act as negative regulator of c-Src. Towards this end, we co-expressed c-Src with FLAG-MPP2 or as control with HA-tagged Mystique, a PDZ protein known to interact with the cytoskeleton of epithelial cells [18]. We then investigated phosphorylation of Y416 in c-Src using anti-pY416Src antibody in total cell lysates by Western blot analysis. We observed a consistent and marked decrease of Src Y416 phosphorylation when co-expressed with MPP2 but not when co-expressed with a control vector or with HA-Mystique (Fig. 4D). The average reduction of the Src Y416 phosphorylation from three experiments was 57% (Fig. 4D, bar diagram). As co-expression of MPP2 with c-Src has the tendency to reduce protein levels of c-Src, we quantified the reduction of Src activity relative to its protein levels. Consistent with the reduced kinase activity of c-Src when co-expressed with MPP2, we also observed decreased overall tyrosine phosphorylation in extracts of cells co-expressing c-Src and MPP2 (Fig. 4E).

A conserved tyrosine residue in MPP2 as a putative regulator of the interaction between MPP2 and c-Src

The crystal structure of the SH3-GuK module of PSD95/DLG4 highlighted the importance of the hinge/HOOK region for protein

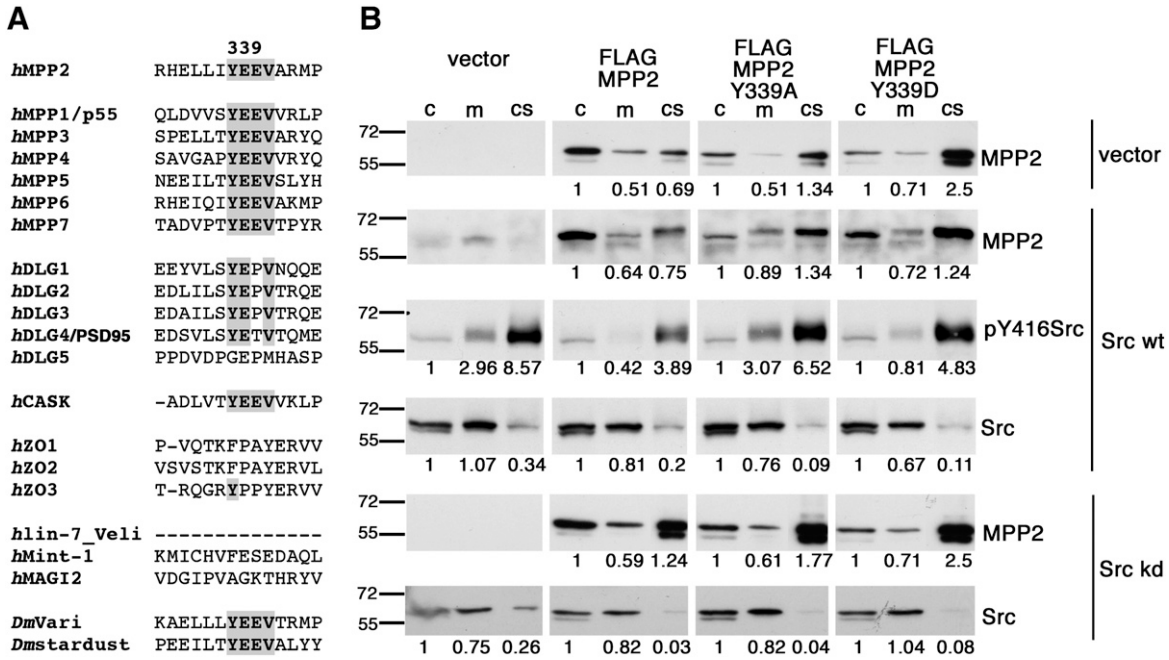

Fig. 5 – A conserved tyrosine residue in MPP2 is a putative regulator of the interaction between MPP2 and c-Src. (A) Alignment of amino acid sequences of MAGUKs around Tyr339 in the hinge/HOOK region of MPP2. Conserved residues are in bold and highlighted in grey. (B) FLAG-MPP2 or FLAG-MPP2Y339A or FLAG-MPP2Y339D were expressed in HEK293 cells together with HA-Src wt or HA-Src kd. Serum-starved cells were fractionated and fractions (c cytosolic, m membrane, cs cytoskeleton) analysed by Wb with antibodies against the proteins indicated to the right of the panels. Quantifications of signal intensities are integrated pixel densities relative to the cytosolic fraction.

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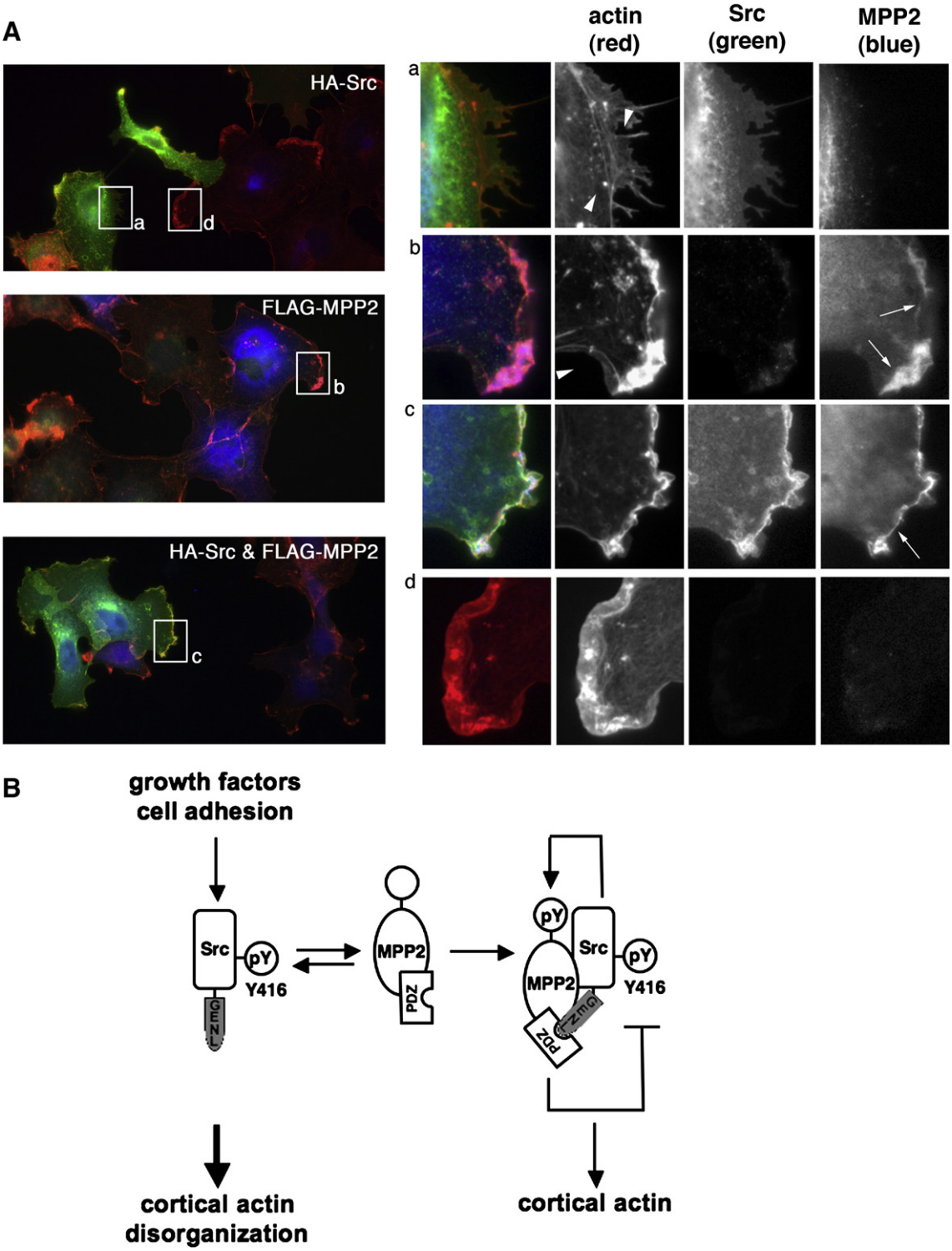

Fig. 6 – MPP2 expression prevents c-Src-promoted disorganisation of the cortical actin cytoskeleton. (A) COS-7 cells were transfected with expression vectors for HA-Src, FLAG-MPP2, or both proteins together. The actin cytoskeleton was visualised with Texas-red-conjugated phallodin (red), and HA-Src (green) and FLAG-MPP2 (blue) with specific antibodies against c-Src and MPP2, respectively. Higher resolution images in panels a, b, c and d are 4-fold magnifications of framed areas. Since c-Src was not overexpressed in b, we used longer exposure times and thereby visualised endogenous c-Src in a dot-like pattern. (B) Model of c-Src MPP2 interaction. Growth factor stimulation or adhesion to the substratum leads to c-Src activation. Activated c-Src interacts and phosphorylates MPP2, which strengthens c-Src-MPP2 interaction. Interaction of c-Src with MPP2 restricts c-Src function by decreasing its kinase activity in a spatially restricted manner.

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conformation. A central Y residue in the hinge/HOOK region in PSD95 is Y523 [16,17]. It fits into the hydrophobic core of the SH3 domain [16], thereby stabilizing the SH3-Guk intramolecular interaction. The corresponding Y residue in MPP2 is Y339 in the YEEV motif (Fig. 1A), which scored highest in MPP2 by computer assisted phosphorylation site screening using NetPhos software (www.cbs.dtu.dk/services/NetPhos/). Y residues analogous to Y339 are highly conserved in MAGUKs (Fig. 5A). We hypothesised that interfering with Y339 either by phosphorylation or by mutation could lead to an altered behaviour of MPP2 in cells. To address the functional significance of Y339 and the possibility of its phosphorylation by c-Src, we substituted Y339 by A (MPP2Y339A) or by D (MPP2Y339D). We first investigated the effects of these mutations on c-Src-dependent Y phosphorylation of MPP2. Y phosphorylation was increased in both mutants compared to wild-type MPP2 (data not shown), indicating that Y339 was not the main target of c-Src-dependent kinase activity and that other/additional residues must be phosphorylated.

A and D substitutions of Y339 enhanced association of over-expressed MPP2 with the cytoskeleton (Fig. 5B, vector control) whereas co-expression of HA-Src wt did not markedly affect MPP2 localisation. However, HA-Src wt shifted the electrophoretic mobility of MPP2 in the cytoskeleton and membrane fraction. In contrast, co-expression of HA-Src kd led to increased MPP2 in the cytoskeleton fraction but not to a shift in electrophoretic mobility.

In agreement with the reduction of Src kinase activity by MPP2 described above (Figs. 4D and E), co-expression of wild-type MPP2 in HA-Src-expressing cells led to decreased Src activity in membranes and the cytoskeleton (Fig. 5B). The inhibitory effect of MPP2 was diminished when Y339 was substituted with A, indicating that Y339 is involved in the negative regulation of c-Src by MPP2.

MPP2 expression prevents the disorganisation of the cortical actin cytoskeleton by overexpressed c-Src

Co-expression of MPP2 in HA-Src-expressing cells reduced Src activity in the membrane and cytoskeleton fractions (Fig. 5B) as well as overall, Src-dependent tyrosine phosphorylation in cells (Figs. 4C and D). Low expression of a c-Src mutant with a non-functional PDZ ligand from a retroviral vector in MCF-10A cells altered cell morphology and disturbed the cortical cytoskeleton [5]. Similarly, strong overexpression of plasmid-derived HA-Src caused disorganisation of the cytoskeleton of COS-7 cells indicated by the lack of actin-rich lamella and the appearance of a dentate morphology (Fig. 6, subpanel a, arrowheads). Since overexpression of MPP2 reduced Src kinase activity in membranes and the cytoskeleton as well as overall tyrosine phosphorylation we tested whether MPP2 could act as inhibitor of c-Src to restrict c-Src kinase-dependent disorganisation of the cytoskeleton. Indeed co-expression of MPP2 in HA-Src-expressing cells prevented Src-dependent disorganisation of the cortical actin cytoskeleton (Fig. 6, subpanel c) as indicated by the presence of regular cortical membrane ruffles similar as those in non-transfected cells (Fig. 6, subpanel d). Both overexpressed proteins, MPP2 (Fig. 6, subpanels b and c, arrows) and HA-Src, co-localised in these structures. These data show that overexpressed MPP2 co-distributes with c-Src to the cell cortex and inhibits Src functions that would lead to the disorganisation of the cytoskeleton.

Discussion

The identification of AF-6 as a PDZ protein regulating c-Src activity in cells by direct interaction [6] highlighted the effect of PDZ proteins as a novel principle of restricting the activity of the c-Src kinase. We have identified here an interaction between c-Src and another PDZ domain protein, namely Membrane Protein Palmitoylated 2 (MPP2). The aim of this work was to characterise the interaction between c-Src and MPP2 and to investigate the localisation and function of MPP2 in epithelial cells. We found that endogenous MPP2 associated with filamentous structures in the basal cytoskeleton, where it partially co-localised with microtubules and c-Src. c-Src interacted with MPP2 in cells, and c-Src kinase activity increased MPP2 phosphorylation and its interaction with c-Src. Conversely, overexpressed MPP2 reduced c-Src kinase activity and reduced overall c-Src-mediated tyrosine phosphorylation in cells. Consistent with this inhibitory effect of MPP2 on c-Src kinase activity, overexpression of MPP2 prevented cortical actin cytoskeleton disorganisation in lamellipodia promoted by overexpressed c-Src. We propose that MPP2 and c-Src constitute a protein complex to control c-Src activity and function.

c-Src interacts with the PDZ proteins AF-6 [6], a junctional adhesion protein and Ligand-of-Numb protein X1 (LNX1) [10], a ubiquitin ligase. Here we show the interaction of c-Src with the PDZ protein MPP2, a Membrane-Associated Guanylate Kinase. Combined, these findings indicate that Src kinases are regulated by diverse PDZ proteins. While the AF-6-c-Src interaction mainly occurs in regions of cell–cell contact, MPP2 and c-Src interacted in the basal cytoskeleton and at the leading edge of migrating cells. These data suggest that the specificity of c-Src regulation through PDZ protein interaction is determined by the spatial restriction of the PDZ protein. Consistently, knock-down of AF-6 in epithelial cells impairs c-Src recruitment to epithelial cell–cell contacts [6]. However, overexpression of a c-Src mutant with impaired PDZ protein binding capability results in morphological alterations not only in regions of cell–cell contact but also in the lamellipodial cytoskeleton [5]. Our data now indicate that MPP2 is a regulator of c-Src in lamellipodia, where it co-localised with c-Src and prevented cytoskeleton disorganisation induced by overexpressed c-Src. This suggests that MPP2 controls c-Src activity in lamellipodia to allow for proper actin polymerisation-dependent lamellipodia formation.

How is MPP2 targeted to the cytoskeleton, where we found both endogenous and overexpressed MPP2? The related MAGUK Dlg is targeted to the subcortical network by its hinge/HOOK region [19]. Our data support such a model also for MPP2 because mutation of the conserved Y339 residue in the hinge/HOOK region of MPP2 – corresponding to regulatory Y523 of PSD95/Dlg4 [16,17] – promoted its translocation to the cytoskeleton. Intriguingly, co-expression of kinase-inactive c-Src along with MPP2 enhanced the accumulation of MPP2 in the cytoskeleton. One possible explanation for this finding is that MPP2 phosphorylated on multiple Y residues is targeted for degradation instead of being recruited to the cytoskeleton. This could be analogous to the enhanced targeting of serine-phosphorylated Dlg for degradation by the Human Papillomavirus E6 oncoprotein [20]. Hence, c-Src-dependent phosphorylation of MPP2 could be part of a negative feedback loop within the circuitry that modulates c-Src activity by MPP2.

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The affinity of PDZ domains for their ligands is generally rather low with dissociation constants in the micromolar range [21]. Thus, additional protein–protein interaction domains present in MAGUKs and c-Src could enhance the avidity of the MPP2–c-Src interaction. Moreover, the interaction between PDZ proteins and their ligands can be controlled by the phosphorylation of serine residues [22,23]. Our data showing that Y phosphorylation enhances the interaction between MMP2 and c-Src indicate that c-Src-dependent tyrosine phosphorylation plays a relevant role in regulating the interaction between the PDZ protein MPP2 and its binding partners.

By NetPhos analysis, we identified 6 putative phosphorylation sites (Y115, Y339, Y378, Y399, Y416 and Y422). Except for Y115 and Y339, all potential tyrosine residues that NetPhos identified lie in the guanylate kinase domain. Since mutating Y339 still resulted in MPP2 phosphorylation, we would predict that c-Src phosphorylates other residues in the guanylate kinase domain. Indeed we only found full-length MPP2 containing the guanylate kinase domain phosphorylated but not a truncation that encompasses Y115 only (data not shown). Hence, association of MPP2 with c-Src and translocation to the cytoskeleton may be regulated by pY-SH2 domain interaction. Alternatively, tyrosine phosphorylation may coordinate a change in conformation in MPP2 that facilitates protein–protein interactions. The latter possibility is supported by structural data that unravelled the key role of the hinge/HOOK region in determining the ternary structure of the SH3-GuK module of Dlg4/PSD-95 [16,17]. Whether Y339 phosphorylation could lead to domain displacement [24,25] and hence would represent another example of a phosphorylation-sensitive interaction in the context of the consensus site D/ELxxxYxD/ExMD/E [26], will require further studies.

Wild-type MPP2 but not the Y339A mutant was able to effectively restrict c-Src, indicating a role of this conserved Y residue in c-Src regulation. At present, it is unclear which kinase would phosphorylate Y339 and its analogs in other MAGUKs and to what extent such a phosphorylation would occur. According to the model for Dlg4/PSD95, however, Y339 would stabilize intra- or intermolecular interactions of SH3 with GuK domains. If Y339 is mutated or phosphorylated, this stabilization would be lost and other interactions could be preferred or permitted, for example due to an open conformation of MPP2 resulting in a stronger association with the cytoskeleton.

A key function of MAGUKs is the establishment and maintenance of polarity in epithelial tissues to allow tissue homeostasis and to prevent aberrant outgrowth of single cells [7,27,28]. MPP2 could contribute to this shared function of MAGUKs by restricting c-Src activity in the cortical and basal cytoskeleton of lamellipodia, in line with the PDZ proteins AF-6 and LNX-1 in cell–cell contacts and membrane ruffles, respectively [6,10].

Acknowledgments

The authors thank Gerald Radziwill for critically reading the manuscript and for helpful discussions. We thank Rafael Fritz and Julia Dennler for providing cell extracts and Daniela Kaul and Josephine Purps for technical assistance. We thank Rosemary O'Connor, University of Cork, Ireland, for providing the HA-Mystique construct. The authors are grateful to the staff of the Centre for Microscopy and Image Analysis of the University of Zürich for their support. This work was supported in part by a grant of the Swiss National Science Foundation to K. M.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.07.028.

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