Myopodin isoforms alter the chemokinetic response of PC3 cells in response to different migration stimuli via differential effects on Rho-ROCK signaling pathways

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The gene encoding myopodin, an actin binding protein, is commonly deleted in invasive, but not in indolent, prostate cancers. There are conflicting reports on the effects of myopodin expression on prostate cancer cell migration and invasion. The recent recognition that myopodin is expressed as four different isoforms further complicates our understanding of how this potentially important invasive prostate cancer biomarker affects tumor cell migration and invasion. We now show that myopodin affects the chemokineti­c, rather than the chemotactic, properties of PC3 prostate cancer cells. Furthermore, all myopodin isoforms can either increase or decrease PC3 cell migration in response to different chemokinetic stimuli. These migration properties were reflected by differences in cell morphology and the relative dependence on Rho-ROCK signaling pathways induced by the environmental stimuli. Truncation analysis determined that a unique 9-residue C-terminal sequence in the shortest isoform and the conserved, PDZ domain-containing N-terminal region of the long isoforms both contribute to the ability of myopodin to alter the response of PC3 cells to chemokinetic stimuli. Matrigel invasion assays also indicated that myopodin primarily affects the migration, rather than the invasion, properties of PC3 cells. The correlation between loss of myopodin expression and invasive prostate cancer therefore reflects complex myopodin interactions with pathways that regulate the cellular migration response to diverse signals that may be present in a tumor microenvironment.

Materials and methods

Cells and reagents

PC3 and NIH 3T3 cells were provided by David Hoskin (Dalhousie University). Cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C in 5% CO2. The anti-myopodin antibody (Abcam) recognized residues 566–585, which is located in the conserved region present in all myopodin isoforms. Horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch), anti-myosin light chain and phosphorylated myosin light chain antibodies (Cell Signaling), anti-Rho A antibody (Cell Signaling), Y-27632 (Sigma), puromycin (Invitrogen), Alexa555-conjugated Phalloidin (Invitrogen), Sequabrene (Sigma), restriction enzymes (NEB biolabs), Matrigel (BD Bioscience) and NSE23766 (EMD Millipore) were purchased from the indicated commercial sources.

Molecular cloning

MYO1, MYO2 and MYO3 in the pcDNA3.1/V5-His-TOPO vector were kindly provided by Jan Gettemans (Ghent University, Belgium). All myopodin isoforms were amplified with Phusion High Fidelity DNA polymerase (Agilent Biotechnologies) and subcloned into the pBMM T retroviral vector. AN-MYO1 was subcloned from the MYO1 template using a forward

Abbreviations: BSA, bovine serum albumin; CM, conditioned medium; FBS, fetal bovine serum; MLC, myosin light chain; MYO, myopodin.
Fig. 1. Myopodin isoforms. Myopodin isoforms are generated via alternative splicing and promoter usage. The upper panel diagrams the arrangement of exons (shaded rectangles) present in the myopodin gene and the location of splice sites. The dashed line above the third exon indicates the location of the alternate promoter used to express the ΔN-MYO1 mRNA. The lower panels indicate the arrangement of exons in the mRNAs encoding the four human myopodin isoforms, all of which share the conserved exon 4 sequence (light gray rectangle). Numbers in exon 4 indicate the total number of amino acid residues present in the isoform. The N- and C-terminal amino acid sequences present at the translational start (arrows) and stop (asterices) sites are indicated above each mRNA. The sequences encoded by the C-terminus of exon 4 are underlined and are conserved in all isoforms, followed by a number indicating the number of residues that separate this conserved sequence from the unique C-terminal sequences of the isoforms (boldface). The site recognized by the polyclonal antiserum is indicated by the arrowhead.

primer that removed the coding sequence for the N-terminal 395 residues. The full-length myopodin (MYO1, MYO2, MYO3 and ΔN-MYO1) and truncated myopodin constructs (ΔN/AC-MYO and ΔC-MYO) were PCR amplified with primers containing BamHI and SalI sites and subcloned into the BamHI and Xhol sites of the pBMN retroviral vector.

Retroviral transduction system
Myopodin constructs in the pBMN vector were transfected into Phoenix cells, a retrovirus packaging cell line, using Polyethyleneimine (Polysciences). After 48h, supernatants were collected and filtered through a 0.45 µm filter. Sequabrene (4% sodium dodecyl sulfate, 0.25% bromophenol blue and 50% glycerol) was added to the viral supernatant prior to infection of PC3 cells. Cells were transduced with the indicated retrovirus vectors for 24h and cultured for 24h with fresh growth medium before selection with puromycin (1 µg/ml) for 3 days. Dead cells and debris were periodically removed by refreezing the monolayers with the selection medium.

Western blotting
Cells were lysed on ice for 20min using RIPA buffer (Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Pierce), and the lysate was sonicated using multiple short bursts with a stainless steel probe sonicator. Samples were centrifuged at 11 000g for 10min at 4°C, and the supernatant protein concentrations were determined using the Bradford protein assay (Bio-Rad). Concentrated protein sample buffer (5% sodium dodecyl sulfate, 0.25% bromophenol blue and 50% glycerol) was added to the cell lysate (1:4 ratio), and supplemented with dithiothreitol (50 mM final concentration) before boiling the lysates. An equal protein load of the cell lysates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5% polyacrylamide), transferred electrophoretically onto polyvinylidene difluoride membranes, blocked with 5% milk, and the membranes were probed with a 1:2500 dilution of polyclonal anti-myopodin antibody and a 1:5000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody. Blots were developed using ECL-Plus reagent (GE Healthcare) and visualized on a Kodak 4000-mm Pro CCD imager.

Microscopy
PC3 cells were cultured on tissue culture treated glass coverslips (Fisher Scientific) and fixed in 3.7% formaldehyde at room temperature for 20min, then permeabilized with 0.25% Triton X-100 in phosphate-buffered saline at room temperature for 10min. Filamentous actin was visualized using Alexa555-conjugated phallolidin (1:40 dilution). Phallolidin-stained cells were viewed and photographed with a Zeiss Axiovert 200M fluorescence microscope. Cells were also fixed with methanol, stained with Wright–Giemsa at the indicated times, and cells were viewed and photographed with Nikon Diaphot-TMD Inverted Microscope.

Transwell migration and invasion assays
Migration and invasion assays were performed using 24-well transwell units with 8 µm porous polycarbonate membranes (BD Falcon). The top chamber was seeded with 0.75×10^5 cells/well and was filled with Dulbecco’s modified Eagle’s medium supplemented with either 0.1% bovine serum albumin (BSA) or 10% FBS, whereas the bottom chamber was filled with 10% FBS/ Dulbecco’s modified Eagle’s medium or NIH 3T3 conditioned medium (CM) as indicated. NIH 3T3 CM was collected from cells after 24 h of serum starvation. Y-27632 or NSC23766 was added to the top and bottom chambers at the indicated concentrations. The 10 µM concentration of Y-27632 is generally considered specific for ROCK, although protein kinase C-related kinase (PRK), another Rho effector kinase, is also inhibited in vitro at these concentrations (17). Cells seeded on top of the insert membrane were incubated for 24h or 12h in the presence of FBS or NIH 3T3 CM, respectively. Following incubation, cells were fixed with methanol, stained with Giemsa or 4′,6-diamidino-2-phenylindole for 10 min, and five random fields were imaged with a 20x objective, using duplicate or triplicate filters for each sample. The average number of cells per field that transmigrated through the membrane was quantified using ImageJ software. For invasion assays, the transwell filters were coated with a thin layer (40 µl) of Matrigel before seeding the cells. The rest of the experimental procedures were the same as in the migration assay. For invasion assays, both the numbers of cells that invaded and the percent cell invasion (i.e. number of cells invading through the Matrigel/number of cells migrating through the filters × 100) are presented. The results of transwell migration and invasion assays were expressed as the mean ± SEM (standard error of the mean) of three independent studies performed in duplicate, or as the mean ± SD (standard deviation) from a representative experiment (n = 3) conducted in triplicate. Statistical significance was performed by a two-tailed Student’s t-test.

Activated Rho-GTP pulldown assay
RhoA activity was examined using the RhoA activation assay Biochem kit (Cytoskeleton) according to the manufacturer’s instructions. Briefly, transduced PC3 cells in 10cm dishes were serum starved for 2 days prior to treatment with 10% FBS, and cell lysates were harvested at the indicated times. About 800 µg of each lysate were incubated with rhotein beads for 1 h at 4°C. After washing the beads, proteins were released from the beads by boiling with protein sample buffer and analyzed by western blotting using anti-Rho A antibody.

Results

Opposing effects of myopodin on cancer cell migration under different chemoattractant conditions
Conflicting reports on whether myopodin promotes or inhibits PC3 cell migration (6,8–10) were obtained under different chemotactic conditions, using either NIH 3T3 CM or 10% FBS as chemoattractants. To determine the contribution of different chemoattractant conditions on cell migration, PC3 cells, an invasive prostate cancer cell line expressing low levels of endogenous myopodin (6), were either mock-transduced or transduced with a retrovirus vector expressing ΔN-MYO1. Due to the phenotypic heterogeneity of PC3 cells, ΔN-MYO1 expression was quantified by microscopic examination of stained filters.

The inherent motility of mock-transduced PC3 cells was greatly influenced by the chemoattractant conditions, and ΔN-MYO1 could inhibit or enhance cell migration depending on these conditions. CM-induced migration of mock-transduced PC3 cells >5-fold more efficiently than FBS (Figure 2A and 2B). ΔN-MYO1 expression inhibited the response of PC3 cells to CM, decreasing the extent of cell migration by ~40% (Figure 2A and 2B). Conversely, under the weak chemoattractant conditions (i.e. 10% FBS), ectopic expression...
had a greater effect on stimulating cell motility than cell invasion, (Figure 2A) and the number of cells that invaded (Figure 3B). CM case of mock-transduced PC3 cells, CM increased both the motility Matrigel was dependent on the external migration stimulus. In the transwell migration assays, the ability of PC3 cells to traverse the numbers of cells that invaded and the percent cell invasion (i.e. number transmigration assay, where of migration >4-fold, but only when cells were also stimulated by N-MYO1 expression increased the extent of the ~80 kDa△N-MYO1 differentially alters the migration and morphology of PC3 cells in response to different chemoattractant conditions. (A) PC3 cells were transduced with retroviral vectors encoding △N-MYO1 or mock transduced, cells were cultured with 0.1% BSA in the upper compartment and either 10% FBS or NIH 3T3 CM in the lower compartment of the Boyden chambers, and the number of cells that migrated across the transwell membranes was quantified. Results are reported as the number of cells migrated per microscopic field relative to mock-transduced cells ± SEM from three independent experiments performed in duplicate. Statistically significant differences between paired samples are indicated (*P < 0.05). (B) Representative images, at 100× final magnification, of Giemsa-stained △N-MYO1- and mock-transduced cells on the lower side of a transwell membrane from a representative experiment as described in panel A. (C) Mock-transduced PC3 cells under the indicated chemoattractant conditions were visualized by differential interference contrast (DIC) microscopy, and by fluorescence microscopy of 4',6-diamidino-2-phenylindole and AlexaFluor555-conjugated phalloidin stained cells. Scale bar = 20 µm.

Myopodin stimulates the chemokinetic, rather than chemotactic, response of PC3 cells to FBS
To investigate the basis for the weak migration response of PC3 cells to FBS and the ability of myopodin to promote cell motility under these conditions, the migration assays were repeated using different combinations of FBS and BSA in the upper and lower compartments of the Boyden chambers. The motility of mock-transduced PC3 cells was the same regardless of whether FBS was present in only the lower chamber, in both the upper and lower chambers, or absent from either chamber (Figure 3A). The same held true when the concentration of FBS in the upper and lower chambers was varied in a checkerboard transmigration assay, where △N-MYO1 stimulated cell migration to approximately the same extent in the absence or in the presence of either negative or positive gradients (Supplementary Table 1, available at Carcinogenesis Online). Although in some experiments FBS alone modestly increased (~2-fold) the migration of mock-transduced PC3 cells (data not shown), △N-MYO1 expression increased the extent of migration >4-fold, but only when cells were also stimulated by FBS (Figure 3A). In PC3 cells that are activated by FBS, myopodin, therefore, promotes a chemokinetic response rather than a chemotactic response to a signaling concentration gradient.

Myopodin affects PC3 cell invasion primarily by altering cell migration
The transwell assays were repeated in the presence of Matrigel to determine the effects of myopodin on cell invasion, and both the numbers of cells that invaded and the percent cell invasion (i.e. number of cells invading through the Matrigel/number of cells migrating through the filters × 100) were determined. As in the previous transwell migration assays, the ability of PC3 cells to traverse the Matrigel was dependent on the external migration stimulus. In the case of mock-transduced PC3 cells, CM increased both the motility (Figure 2A) and the number of cells that invaded (Figure 3B). CM had a greater effect on stimulating cell motility than cell invasion, resulting in ~50% decrease in the percent cell invasion (Figure 3C). Ectopic expression of △N-MYO1 suppressed PC3 cell migration and invasion in response to CM to approximately the same extent (Figures 2A and 3B), leading to a modest reduction in the percent invasion (Figure 3C). Conversely, 10% FBS failed to stimulate PC3 cell motility (Figure 3A), but ~90% of the mock-transduced PC3 cells that were motile were also invasive (Figure 3C), a reflection of the inherent invasiveness of this cell line. Expression of △N-MYO1 promoted cell migration in response to FBS by ~4-fold (Figures 2A and 3A) while promoting cell invasion ~3.5-fold (Figure 3B), resulting in an ~20% decrease in the percent invasion (Figure 3C). Therefore, even under conditions that differentially affect cell chemokinetic activity, myopodin expression correlates with a decrease in the percentage of the cells that can penetrate through extracellular matrix.

All human myopodin isoforms affect cell migration
The above studies, and the majority of previous studies on myopodin, used the △N-MYO1 isoform. Moreover, the siRNAs used for the knockdown studies targeted all four isoforms of myopodin (9), not just the △N-MYO1 isoform used in the ectopic expression studies (6,8). The three long isoforms contain a conserved N-terminal region that includes a PDZ domain that is absent from the naturally occurring △N-MYO1 isoform (16). To determine whether the different MYO isoforms exhibit similar effects on cell migration and invasion, the different isoforms were ectopically expressed in PC3 cells. All of the isoforms were expressed at similar levels as determined by western blotting using a polyclonal antiserum raised against epitopes present in the conserved exon 4 (Figure 4A), although the △N-MYO1 isoform appeared at higher levels in some blots (compare Figures 4A and 5B). Western blots also detected a slower migrating ~110 kDa version of the ~80 kDa △N-MYO1 isoform, which presumably reflects an undefined posttranslational modification. When examined using the transwell migration assay, all of the isoforms modestly suppressed PC3 cell migration under CM conditions but substantially enhanced cell migration under FBS conditions at levels similar to those observed with △N-MYO1 (Figure 4B). All myopodin isoforms, therefore, promote or inhibit cell migration, depending on the nature of the migration stimulus.

Multiple regions of myopodin contribute to enhanced cell migration
To define regions in myopodin that alter the chemokinetic response of PC3 cells, a series of myopodin truncation constructs were created that removed the unique N- and/or C-termini present in the different isoforms (Figure 5A). These constructs were expressed at
approximately equivalent levels in transduced PC3 cells, as determined by western blotting (Figure 5B). Although ΔC-MYO produced a single polypeptide band, as did the parental MYO3, the ΔN-MYO3 and ΔC/ΔN-MYO constructs expressed two versions of the myopodin protein, suggesting that sequences encoded by exons 1, 2 and the 5’-terminal part of exon 3 alter either the posttranslational modification or processing of myopodin. The naturally occurring ΔN-MYO1 isoform and the artificial ΔN-MYO3 construct both increased PC3 cell migration in response to FBS and modestly decreased migration in response to CM, to approximately the same extent as the full-length MYO1, MYO2 and MYO3 isoforms (Figure 5C). All of these changes were statistically significant (P < 0.05, n = 3). The presence or absence of the N-terminal PDZ domain, therefore, does not affect the ability of myopodin to regulate the cell response to chemokinetic stimuli.

The observation that MYO1, MYO2 and MYO3 all have similar effects on PC3 cell migration implies that the unique C-termini present in these isoforms are not the determinants of chemokinetic regulation. To determine whether these unique C-termini are also dispensable, a C-terminal truncation was created (ΔC-MYO) that contained the sequences shared by all the long MYO isoforms but was lacking the unique C-termini present in each of these isoforms. The ΔC-MYO construct had the same statistically significant effect (P < 0.05, n = 3) on PC3 cell migration as the full-length constructs under FBS conditions (Figure 5C). Although results did not reach statistical significance, ΔC-MYO also suppressed migration under CM conditions in all three replicates of the experiment. The C-termini of the long MYO isoforms, therefore, exert little, if any, affect on regulating chemokinesis. Interestingly, whereas deletion of the C-terminal 9 residues from MYO1 had no effect on cell migration, removal of the same sequence from ΔN-MYO1 severely impaired the ability of this isoform to promote cell migration in the presence of FBS or to inhibit the migration induced by CM (Figure 5C). Thus, the conserved N-termini present in the three long myopodin isoforms and the unique C-terminus of ΔN-MYO1 individually contribute to the chemokinetic effects of myopodin.

Myopodin-stimulated cell migration is partially dependent on a Rho-ROCK signaling pathway

The rounded versus elongated morphologies induced by FBS and CM (Figure 2C) were suggestive of differential activation of the Rho family of small GTPases associated with cytoskeletal dynamics and cell migration (18,19). Pharmacologic inhibitors were, therefore, used to explore the relative contributions of these pathways to the chemokinetic effects of myopodin. The Rho-associated coiled-coil forming serine/threonine kinase (ROCK) is a downstream effector in the Rho pathway (20). Addition of a ROCK inhibitor (Y-27632) to the transwell migration assay abrogated the increased cell migration in response to CM, of both mock-transduced and ΔN-MYO1-transduced PC3 cells (Figure 6A) at statistically significant levels (P < 0.05, n = 3). There was no additive effect of myopodin and ROCK inhibitor on PC3 cell migration. CM, therefore, promotes PC3 cell migration by activating the Rho signaling pathway, and myopodin partially suppresses migration via its effects on activation of this pathway. Under FBS conditions, the ROCK inhibitor had no effect on the inherent migration activity of PC3 cells, but it did decrease cell migration induced by ΔN-MYO1 by ~40% (Figure 6A) at statistically significant levels (P < 0.05, n = 3). Direct examination of Rho activation under FBS conditions using a Rho-GTP pulldown assay indicated mock-transduced PC3 cells respond to FBS by modestly activating Rho, but expression of myopodin resulted in a 4-fold increase in Rho activation under these conditions (Figure 6B). Myopodin expression, therefore, increases activation of the Rho pathway in response to FBS, and inhibiting a downstream effector of this pathway reduces the level of myopodin-stimulated cell migration.

To further examine why the ROCK inhibitor only partially reduced the motility response of PC3 cells to myopodin expression under FBS conditions, dose–response assays were conducted on cells expressing ΔN-MYO1 and another myopodin isoform, MYO3. The ROCK inhibitor had no effect on the migration activity of mock-transduced PC3 cells, but it decreased both ΔN-MYO1- and MYO3-induced cell migration by ~40% in a dose-dependent manner (Figure 6C). Incomplete inhibition of ROCK activity was an unlikely explanation for the partial reduction in cell motility, since phosphorylation of the myosin light chain, a downstream target of ROCK (20), was reduced for the partial reduction in cell motility, since phosphorylation of the myosin light chain, a downstream target of ROCK (20), was reduced for the partial reduction in cell motility, since phosphorylation of the myosin light chain, a downstream target of ROCK (20), was reduced for the partial reduction in cell motility, since phosphorylation of the myosin light chain, a downstream target of ROCK (20), was reduced for the partial reduction in cell motility, since phosphorylation of the myosin light chain, a downstream target of ROCK (20), was reduced.
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the roles of recently identified myopodin isoforms in this process,

Discussion

Why loss of myopodin expression correlates with tumor invasion, and
the roles of recently identified myopodin isoforms in this process,
are presently unclear. There are also conflicting reports on whether
myopodin affects PC3 cell migration (6,8), and whether myopodin
suppresses or promotes cell invasion (7,9,10). This study provides an
explanation for these anomalies. Results indicate that myopodin alters
the motility response of PC3 cells to external stimuli that differen-
tially activate Rho/ROCK signaling pathways. Under conditions that
substantially increase ROCK-dependent PC3 cell migration, myopo-
din partially suppresses this motility response. Conversely, myopodin
can dramatically increase PC3 cell motility in the presence of a weak,
external migration stimulus, a response that is mediated, at least par-
tially, via activation of Rho/ROCK signaling pathways. Results also
indicate that the effects of myopodin on cell invasion reflect changes
to the chemokinetic, rather than the chemotactic, response of PC3
cells to external stimuli, and that all of the myopodin isoforms exert
a similar effect on the cellular response to chemokinetic stimuli.
Myopodin can, therefore, differentially regulate signaling pathways
likely to be triggered by diverse signals present in a tumor microen-
vironment, thereby altering the invasion potential of tumor cells.

The unappreciated ability of myopodin to exert opposing effects
on the response of PC3 cells to different external stimuli clarifies
confusion over whether myopodin promotes or suppresses cell
motility and invasion. Overexpression or RNAi knockdown of
myopodin have both been reported to suppress PC3 invasion (6,8,9).
The former studies used CM as a chemoattractant, whereas the latter
used FBS. As we now show, both of these prior observations are
correct; myopodin suppresses the motility of PC3 cells in response
to CM but promotes migration under FBS conditions (Figure 2). Transwell migration assays indicated that myopodin mediates these
effects by altering the chemokinetic response of PC3 cells to FBS,
which refers to a change in cell motility in response to soluble factors
in the absence of a chemical gradient (23). The profound effects of
myopodin on chemokinesis were also reflected in the overall invasion
potential of the cell population through Matrigel. In the presence
of CM, myopodin suppressed both cell migration and invasion by
~40–50%, whereas myopodin increased migration and invasion by
3.5- to 5-fold in response to FBS stimulation (Figure 3). Myopodin
therefore affected migration and invasion to approximately the same
degree, regardless of whether it was promoting or inhibiting these
processes, suggesting that myopodin affects cell invasion primarily
by altering cell motility. The basis for these effects remains to be
determined, but one possibility is that myopodin affects the balance
between intrinsically random versus directionally persistent PC3
cell migration. A similar effect was noted previously in PC3 cells
in response to insulin-like growth factor-1 stimulation, where Rho
C activity converts persistent linear migration to random, non-linear
migration (24). Regardless of the actual mechanism, the present
results clearly indicate that myopodin can have diametrically opposed
effects on the response of PC3 cells to different external stimuli.

Truncation analysis indicated that sequences in the extended
N-terminus conserved in the long myopodin isoforms and the unique
9-residue C-terminus present in the ∆N-MYO1 isoform both contrib-
tute to the migration effects of myopodin (Figure 5). It was only when
both termini were deleted (the ∆C/∆N-MYO construct) that a dra-
matic reduction in the cell motility response was observed. Previous
interaction and deletion analyses using the human ∆N-MYO1 iso-
form identified several regions that interact with a number of cellular
partners, including zyxin, integrin-linked kinase (ILK), α-actinin and
filamin (7,8,16). The mouse homolog of this isoform also contains
an actin binding site (15). However, all of these interaction sites are
contained within the central conserved region of myopodin, which is
retained in the ∆C/∆N-MYO construct. No interaction studies have
been performed with the extended myopodin isoforms that contain
the N-terminal PDZ domain, and no studies have specifically used
the unique C-termini of the myopodin isoforms as bait, two regions
we have shown to be important for the migration phenotypes
of myopodin-expressing cells. Further interaction studies based on
the myopodin termini may therefore identify additional partners impor-
tant for regulating cancer cell migration.

Much of the confusion on whether myopodin promotes or inhibits
cell migration reflects the differential effects of generic migration
stimuli on the inherent motility of PC3 cells. It is clear that PC3 cells
respond to a diverse range of internal and external signals via differen-
tial activation of both Rac and Rho signaling pathways (21,22,24–29). As
we now show, CM is a potent stimulator of PC3 cell migration and
induces an elongated morphology in all cells, while cells tended to
assume a rounded morphology and displayed low motility in response
to 10% FBS (Figure 2). These differences in morphology and migration
are reminiscent of changes that occur when the relative balance of

Fig. 4. All myopodin isoforms exert similar differential effects on the
migration of PC3 cells in response to different chemoattractant conditions.
(A) PC3 cells were transduced with retrovirus vectors expressing the
indicated myopodin isoforms or mock-transduced, and myopodin expression
levels were determined by western blotting using a polyclonal anti-myopodin
antiserum. The migration of molecular mass standards is indicated on
the left. The asterisk indicates a cross-reacting host cell polypeptide. (B)
The migration of PC3 cells, mock-transduced or transduced with vectors
expressing the indicated myopodin isoforms, through transwell membranes
in response to the indicated migration stimulus (CM or FBS) was determined
as described in Figure 2. Results are reported as the mean fold change in cells
migrated relative to mock-transduced cells ± SEM from three independent
experiments performed in duplicate. Statistically significant differences
between paired samples are indicated (*P < 0.05; **P < 0.01).

Carcinogenesis Online). Thus, the ability of myopodin to promote
PC3 cell migration in the presence of weak chemokinetic stimuli
reflects activation of a Rho-ROCK signaling pathway, but additional
ROCK-independent pathways that do not require Rac activation are
also probably involved.
activated forms of the Rho family of small GTPases is shifted. For instance, increasing concentrations of activated Rac GTPase promotes symmetrical lamellipodia formation and decreases directional migration, whereas increasing activation of Rho-GTPase induces cell polarization and increased lamellipodia at the leading cell edge to promote persistent directional migration (20,30). The observation that inhibiting ROCK, a downstream effector of Rho, dramatically impaired the ability of CM to stimulate cell migration (Figure 6A) indicates CM activates Rho pathways in PC3 cells and is consistent with the elongated morphology and high motility of cells under these conditions. Conversely, FBS had only a modest effect on Rho-GTP levels in mock-transduced cells (Figure 6B), this level of activation was insufficient to promote cell migration (Figure 6A), and a ROCK inhibitor had no effect on PC3 cell migration in the presence of FBS (Figure 6A). Thus, substantial differences exist in the complex signaling changes induced in mock-transduced PC3 cells by CM and FBS, at least some of which involve differential effects on Rho signaling pathways.

Most importantly, the effects of myopodin on PC3 cell migration in response to the different external stimuli reflect altered activation of ROCK-dependent and ROCK-independent signaling pathways. Under conditions that promote a ROCK-dependent chemokinetic response in PC3 cells (i.e. CM), myopodin modestly suppresses cell migration. Why myopodin only partially inhibits the ROCK-dependent activation of PC3 cells by CM is unclear. Previous studies, using clonal isolates of myopodin-expressing PC3 cells, showed either no effect of myopodin on cell migration or ~50–80% decrease in migration we observed (Figures 2A and 6A). Myopodin, therefore, displays a variable ability to partially block the potent activation of ROCK-dependent migration stimulated by CM. In contrast, a weak migration stimulus (i.e. FBS) renders cells responsive to a myopodin-dependent chemokinetic response that correlates with ~4-fold increases in Rho-GTP levels and cell migration (Figure 6A and 6B), and the migration response is reduced by ~40% in the presence of a ROCK inhibitor (Figure 6C and 6D). Myopodin can, therefore, promote cell migration via activation of the Rho-ROCK signaling pathway. Based solely on the ROCK inhibitor results, we cannot exclude the possible additional involvement of the Rho effector kinase PRK that is also inhibited, at least in vitro, by similar concentrations of the Y-27632 inhibitor (17). However, the cell morphology changes are certainly consistent with well-described alterations to the Rho-ROCK signaling pathway. Rho C activation has also been shown to promote PC3 cell migration via increased directional motility toward a chemoattractant and decreased linear random migration (24,25). Such is not the case for myopodin, which clearly promotes a chemokinetic, rather than a chemotactic, response in PC3 cells (Supplementary Table 1, available at Carcinogenesis Online).

The ROCK inhibitor results also suggest that the motility response of PC3 cells to myopodin is not entirely ROCK-dependent, since the inhibitor effectively eliminated phosphorylation of the myosin light chain target of ROCK while migration was only inhibited by 40% (Figure 6D). Coupled with the observation that inhibiting Rac activation has no effect on the stimulatory effect of myopodin on cell migration (Supplementary Figure 1, available at Carcinogenesis Online), it is now apparent that the effects of myopodin on cell migration involves more that just alteration of the complex interplay between the Rac and Rho signaling pathways. The mouse, chicken and rabbit homologs of myopodin have actin binding, polymerization and bundling activity (15,31,32). Myopodin also binds to integrin-linked
kinase and zyxin, both of which are important players in focal adhesion complexes (7,8). Thus, the effects of myopodin on cell migration may well reflect additional Rho- and Rac-independent changes on the actin cytoskeletal network and/or focal adhesion dynamics.

The strong correlation between invasive tumor development and the loss of expression suggests that myopodin may function as a tumor suppressor. Overexpression of myopodin in a naturally invasive prostate cancer cell line can also reduce the metastatic potential of PC3 cells in vivo (6). A straightforward explanation for this phenotype is the ability of myopodin to suppress cell motility in response to certain external stimuli. The present results, however, caution against such an oversimplified interpretation of the existing data. Diverse signals in the tissue microenvironment promote, inhibit and coordinate the migration of both normal and neoplastic cells. As we show, myopodin can promote or repress cell motility, either or both of which could provide an explanation for the role of myopodin as a tumor suppressor. For example, the coordinated, directional migration of epithelial cells is required to maintain the epithelium (33). If myopodin naturally functions to promote epithelial cell migration, then loss of myopodin expression would result in disruption of the epithelium and associated basal lamina that serve as barriers to tumor cell invasion. The function of myopodin as a tumor suppressor might, therefore, reflect roles in promoting and/or inhibiting cell migration. Further studies are clearly needed to determine how the complex roles of myopodin in regulating the cellular response to external migration stimuli affect tissue homeostasis and tumor cell invasion.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at http://carcin.oxfordjournals.org/

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References


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