MIM-B, a putative metastasis suppressor protein, binds to actin and to protein tyrosine phosphatase δ

Jacquelyn A. WOODINGS¹, Stewart J. SHARP¹ and Laura M. MACHESKY²

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

We have found that MIM-B, a putative metastasis suppressor protein, is implicated in actin cytoskeletal control and interaction with a protein tyrosine phosphatase (PTP). MIM was originally described as a protein whose mRNA was *Missing in Metastasis*, as it was found not to be present in metastatic bladder carcinoma cell lines [Lee, Y. G., Macoska, J. A., Korenchuk, S. and Pienta, K. J. (2002) *Neoplasia* 4, 291–294]. We further characterized a variant of MIM, which we call MIM-B, and which we believe may be a link between tyrosine kinase signalling and the actin cytoskeleton. We have shown, using purified proteins and cell extracts, that MIM-B is an actin-binding protein, probably via a WASP (Wiskott–Aldrich syndrome protein)-homology 2 domain at its C-terminus. We have also found that MIM-B binds to the cytoplasmic domain of receptor PTP δ . Expression of full-length

MIM-B induces actin-rich protrusions resembling microspikes and lamellipodia at the plasma membrane and promotes disassembly of actin stress fibres. The C-terminal portion of MIM-B is localized in the cytoplasm and does not affect the actin cytoskeleton when expressed, while the N-terminal portion localizes to internal vesicles and probably targets the protein to membranes. We postulate that MIM-B may be a regulator of actin assembly downstream of tyrosine kinase signalling and that this activity may explain the involvement of MIM in the metastasis of cancer cells.

Key words: actin, cytoskeleton, metastasis, motility, phosphatase, signalling.

INTRODUCTION

There are many physiological signals that can trigger changes in cell motility and shape. Thus the actin cytoskeleton is controlled by signals from a variety of receptors, including tyrosine kinases, G-protein coupled receptors and integrin-related receptors. One of the main controlling pathways of actin assembly in cells appears to involve Wiskott–Aldrich syndrome protein (WASP)-family proteins, including the five mammalian components (WASP, N-WASP, Scar/WAVE1-3) and the Arp2/3 complex [1]. The Arp2/3 complex consists of seven subunits, two of which are related to actin and thought to participate in the nucleation of new actin filaments off the sides of pre-existing ones [1]. The WASP proteins bind directly to Arp2/3 and activate its nucleation activity. Signals from receptors, including tyrosine phosphorylation either directly [2] or through adapter protein recruitment [3,4], activation of the small GTPases Cdc42 and Rac or changes in membrane phospholipid composition, are thought to activate WASP-family proteins at the plasma membrane. How receptors communicate with specific WASP-family proteins using these different mechanisms is still an area of active research.

Cells contain proteins divergently related to the WASP-family proteins, which also appear to be important regulators of the actin cytoskeleton. WASP is composed of the following modular domains (listed here in consecutive order from the N- to the C-terminus): WH1 (WASP-homology 1), GBD (GTPase binding domain), Pro (proline-rich), WH2 (WASP-homology 2) and CA (central basic plus acidic Arp2/3 binding). WH2 motifs bind to actin monomers [5]. Several proteins in eukaryotes have been identified which contain WH2 homology regions. Most of these proteins are linked to functions in polarity and actin cytoskeletal

regulation. In *Saccharomyces cerevisiae*, for example, verprolin is a WH2-containing protein that interacts with the WASP homologue Las17p/Beel1p [6]. Verprolin regulates polarity, cytokinesis, endocytosis and cytoskeletal organization [7,8]. Another WH2-containing protein is p150Spir, which may be involved in transport from the trans-Golgi network to the plasma membrane [9]. In *Drosophila*, p150Spir (also called SPIRE) is important in polarity [10,11]. A new protein MIM (missing in metastasis) has been identified which contains a C-terminal WH2 motif and is postulated to be an important regulator of the actin cytoskeleton [12].

Receptor protein tyrosine phosphatases (RPTPs), including RPTP δ [13], RPTP α [14] and RPTP β [15], have been linked in many studies to cell motility and the actin cytoskeleton. RPTP δ regulates the chemotaxis of neurons toward a gradient of its own extracellular domain [16,17]. Another related phosphatase, *Drosophila* leukocyte common antigen-related (DLAR), is a *Drosophila* RPTP that is related to the human LAR (leukocyte common antigen-related acetate) as well as to RPTP δ . DLAR binds to the actin cytoskeletal protein Ena [18] and regulates the organization and polarity of basal F-actin in the follicular epithelium of *Drosophila* [19]. DLAR also interacts with the Rac/Rho GEF (guanine nucleotide exchange factor) trio [20]. In addition to being involved with the actin cytoskeleton and motility, RPTPs are also implicated in the regulation of cell-matrix and cell–cell adhesion. For example, RPTP α dephosphorylates and activates Src family kinases, which in turn phosphorylate components of adherens junctions to modulate cell–cell contacts [14].

We describe here a unique protein (MIM-B) containing a WH2 motif at the C-terminus, which we postulate to be involved in cell motility and/or adhesion and for which a fragment of a

Abbreviations used: DLAR, *Drosophila* leukocyte common antigen-related; GST, glutathione-S-transferase; MIM, missing in metastasis; RPTP, receptor protein tyrosine phosphatase; RPTP δ -CD, cytoplasmic domain of RPTP δ ; WASP, Wiskott–Aldrich syndrome protein; WH2, WASP-homology 2.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail L.M.Machesky@bham.ac.uk).

Figure 1 MIM-B domains and constructs

(A) Constructs used in various experiments are shown here, with the various ascribed properties indicated (vesicular localization, RPTP δ -binding and actin-binding). Numbers indicate the amino acid number. (B) Sequence alignment, performed using MACAW, for MIM-B (MIM-2) with the closest homologues in *Drosophila* (dMIM; Flybase ID CG9469) and *C. elegans* (ceMIM; Genbank® AC006676). In this alignment, and in (D), the upper case letters represent linked sequences that show significant homology (as determined by MACAW), whilst the lower case letters represent non-homologous stretches of sequence. (C) Graphic representation of homology between MIM-B and the *Drosophila* and *C. elegans* homologues. (D) Homology between MIM-B and BAIP1-2 (also known as IRS-p53) in the N-terminal portions of both proteins. The function of the N-termini are unknown, but may be used for membrane targeting. (E) Alignment of the WH2 motif of MIM-B with homologous portions of the next three closest relative WH2 motifs from *S. cerevisiae* verprolin (VERP), human WIP and *Acanthamoeba* Actobindin (Acb), and comparison with human WASP WH2 (WAS) below.

close homologue, MIM, has been identified by another group as a metastasis suppressor ([12] and Genbank® AF086645 and KIAA0429). MIM-B binds to actin with a micromolar affinity *in vitro*, using purified protein, and *in vivo* when expressed in COS-7 cells. MIM-B also binds to the cytoplasmic domain of the RPTP δ in the yeast two-hybrid assay and in extracts from COS-7 cells incubated with recombinant RPTP δ . MIM-B localizes to areas of dynamic actin assembly and intracellular vesicular structures and overexpression of MIM-B leads to the assembly of actin-rich protrusions resembling surface ruffles and microspikes. We propose that MIM-B links receptor tyrosine phosphatase signalling with actin cytoskeletal reorganization and provides a potential new link between tyrosine phosphorylation signalling and the actin cytoskeleton.

METHODS

Chemicals and reagents

All chemicals, yeast and bacterial growth media and supplements were purchased from Sigma-Aldrich U.K. unless otherwise stated.

DNA constructs

The following DNA constructs (Figure 1) were designed: for the yeast two-hybrid assays, pYTH9-404-705MIM-B, pYTH9-404-759MIM-B, pACT-II Full length Scar1 and pYTH9-p21-Arc; for the cell biology, pRK5myc-1-520MIM-B, pRK5myc-706-759MIM-B, pRK5myc-565-759MIM-B, pRK5myc-664-759MIM-B, pRK5myc-404-759MIM-B; and for the pulldown assays, pGEX2T-1-520MIM-B, pGEX2T-706-759MIM-B, pGEX2T-404-759MIM-B, pGEX2T-404-705MIM-B, pGEX2T-706-759MIM-B, pGEX2T-664-759MIM-B, pGEX2T-565-759MIM-B and GST-PTP δ -CD (cytoplasmic domain of RPTP δ , a gift from Michel Streuli, Harvard Medical School, Boston, U.S.A.; [13]).

Protein preparation

Glutathione S-transferase (GST)-fused MIM-B fragments were prepared as previously described for WASP-family proteins [25] and using glutathione-agarose beads. The purified protein was cleaved from the beads using thrombin. Thrombin was removed with *p*-aminobenzamidine beads (Sigma) and proteins were used fresh or separated into aliquots and frozen at -80°C .

Actin was purified from rabbit muscle acetone powder by the method of Spudich and Watt [21] and gel-filtered [22] to isolate monomers for polymerization assays. Pyrene actin was prepared as described previously [23] and gel-filtered as above. Fresh actin was used within a week of preparation and pyrene actin was separated into aliquots and stored at -80°C .

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as previously described [24] using a human brain library (Clontech) screened with pYTH9 coding for amino acids 404–705 of MIM-B. Two positive clones were obtained and both were sequenced and found to encode amino acids from position 1347 to the C-terminus of the cytoplasmic domain of the RPTP δ . The interaction was re-tested by transforming RPTP δ and 404-705MIM-B back into Y190 yeast and assaying Gal4 activity using the filter assay [24]. Positive controls were Scar1 and p21-Arc and negative controls were

404-705MIM-B or 404-759MIM-B against Scar1 or RPTP δ -CD and p21-Arc.

Actin binding to MIM-B constructs was tested in a pulldown assay as follows: recombinant MIM-B proteins fused to GST were isolated on glutathione-agarose beads and equilibrated with G-buffer (2 mM Tris/HCl, pH 7.5, 0.2 mM ATP, 0.5 mM dithiothreitol, 2 mM CaCl_2). Beads were then incubated with monomeric actin for 30 min at 4°C . A control tube containing no actin was included. After three washes in G-buffer, the proteins in the supernatant and pellet fractions were separated by SDS/PAGE and stained with Coomassie Blue. A semi-quantitative estimate of the binding of actin to the GST beads was made using the computer programme NIH Image to measure the area and density of each band. A one-site binding curve of actin concentration against the binding ratio of actin/MIM-B fragment was plotted using computer-assisted curve fitting (Prism 2 Graphpad Software). The equation fitted was:

$$Y = B_{\max} \cdot X / (K_d + X)$$

where $B_{\max}$ is the maximum concentration of binding sites (which is the same as the concentration of recombinant protein), K_d is the dissociation equilibrium constant and X is the concentration of actin.

Actin polymerization

Actin polymerization assays were performed as described previously [23]. Briefly, recombinant protein was added to 3 μM actin [5% (w/w) pyrene-labelled] in G-buffer. Polymerization was initiated using $10 \times$ KME (50 mM KCl, 1 mM EGTA, 1 mM MgCl_2) and continued for 7200 s. An excitation wavelength of 370 nm was used and emission was measured at a wavelength of 407 nm using a PTI C60 spectrofluorimeter (Photon Technology International).

RPTP δ binding to MIM-B (pulldown assays)

COS-7 cells (approx. 6×10^6 cells per experiment) were transfected with MIM-B constructs using Fugene 6 (Roche) according to the manufacturer's instructions. After 24 h, cells were placed on ice for 5 min and washed twice with PBS containing protease inhibitors (CLAP: chymostatin, leupeptin, antipain and pepstatin at a final concentration of 0.1 $\mu\text{g}/\text{ml}$ of each and PMSF at 50 μM). Cells were then overlaid with 200 μl of GST-binding buffer [10% glycerol, 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1% (v/v) IgepalCA-630] for a further 5 min before scraping into a tube. After centrifugation for 5 min in an eppendorf centrifuge at 16000 g , cell supernatants were incubated with 30 μl of GST-PTP δ -CD recombinant protein on glutathione-agarose beads for 30 min at 4°C . After 3 washes in GST-binding buffer, the supernatants and pellets were separated on 8% polyacrylamide gels in SDS and analysed by Western blotting.

Cell biology

Transient transfections were performed using Polyfect (Qiagen) for NIH3T3 cells or Fugene 6 (Roche) for COS-7 cells, according to the manufacturer's instructions. Anti-myc antibodies were a gift from Alan Hall (Department of Biochemistry, MRC-LMCB, London, U.K.) and Robert Insall (School of Biosciences, University of Birmingham, U.K.), secondary antibodies were purchased from Jackson Labs (Strattech Scientific Ltd., Soham,

U.K.). Cells were fixed in 4% paraformaldehyde in PBS and mounted in Mowiol with antifade, as described previously [24a].

RESULTS

We identified MIM-B from the NEDO (New Energy and Industrial Technology Development Organization, Japan) human cDNA sequencing project (Genbank® AK027015 and Figure 1A). This cDNA encodes a protein that is predicted to be nearly identical to YGL-1 (Genbank® AF086645) and which has a C-terminal sequence (amino acids 404–759) that is nearly identical to a fragment of YGL-1 [HUGE (Human Unidentified Gene-encoded Large Proteins database, Kasuza, Japan) KIAA0429]. There are three point substitutions between YGL-1 and MIM-B, which probably correspond to sequencing errors, and an insertion of four extra codons in the N-terminal domain of MIM-B. Both cDNAs map to the same locus of chromosome 8 and we propose to call the products MIM and MIM-B. MIM proteins appear to be conserved, with homologues in mouse (results not shown; [25]), *Drosophila* (Figures 1B and 1C), and *Caenorhabditis elegans* (Figures 1B and 1C) but not to our knowledge in *S. cerevisiae* or *Dictyostelium*. Humans appear to have two closely related isoforms, with only 6 amino acids different between YGL-1 and MIM-B. The closest relative to YGL-1 or MIM-B appears to be the insulin receptor tyrosine kinase substrate BAIP1-2 or IRSp53 (Figure 1D), which has limited homology in the N-terminal region. It is interesting to note that MIM-B is also likely to be a substrate for tyrosine phosphorylation, so this homology may be related to interaction with tyrosine kinases or membranes (see below). MIM-B, like MIM, has a WH2 motif at the extreme C-terminus (Figure 1E) which shows a high degree of identity with the WH2 motifs of verprolin, WIP and actobindin. This WH2 is quite different from those found in WASP (Figure 1E) and other WASP-family proteins (results not shown) but both types of WH2 appear to bind with micromolar affinity to monomeric actin ([26,27] and see below).

Since MIM-B had a WH2 motif, we tested whether this motif was functional in binding to actin monomers. We performed pulldown assays using recombinant MIM-B fragments and rabbit muscle G-actin and found that the smallest fragment that would still significantly interact with G-actin corresponded to amino acids 404–759 (Figure 2). Deletion of the WH2 motif to create 404-705MIM-B eliminated the binding, showing that the WH2 motif is necessary for the interaction (Figure 2). It is likely that amino acids near the WH2 motif also contribute to the actin association, or to the overall protein structure and folding, as 664-759MIM-B bound almost no G-actin in a pulldown assay (Figure 2). Semi-quantitative analysis of the binding to G-actin revealed a $0.5 \mu\text{M}$ K_d for 404-759MIM-B, the largest fragment that we were able to produce in *Escherichia coli*. A summary of the constructs tested in this assay and whether we detected an interaction with G-actin is shown in Figure 1(A). These fragments also interacted with G-actin in crude lysates from bovine spleen or from COS-7 cells, as detected with pulldown and Western blot analysis (results not shown). Therefore we conclude that the WH2 motif in MIM-B is functional for binding to actin monomer and MIM-B is thus implicated as a potential regulator of the actin cytoskeleton.

Figure 2 Actin monomers binding to MIM-B

The graph shows actin-binding curves to 404-759MIM-B ($\square$, $n = 3.6$), 404-705MIM-B ($\triangle$, $n = 3$), and 706-759MIM-B ($\circ$, $n = 6$). The binding ratio is the amount of actin/the amount of MIM-B fragment (A/MF) in the pellet fraction. Actin binding to 404-705MIM-B did not increase with actin concentration and is therefore non-specific binding. Actin binding to 706-759MIM-B is at a very low level (see Discussion section). The K_d for actin binding to 404-759MIM-B was calculated as $0.53 \mu\text{M}$.

We further analysed the interaction of MIM-B with actin, using a polymerization assay to determine whether MIM-B sequestered actin monomers, similar to other WH2-containing proteins [27]. Addition of increasing concentrations of MIM-B fragments that contained the WH2 motif slowed the nucleation and elongation of the actin in a similar manner to other WH2 proteins or to profilin. A short fragment containing the WH2 motif (amino acids 664–759) caused approx. half-maximal inhibition at 500 nM (Figure 3A), whereas a longer fragment, 404-759MIM-B, was slightly less effective (Figure 3B). Deletion of the WH2 motif (404-705MIM-B) completely abolished the effect on actin assembly (Figure 3C). Within a concentration factor of two, most constructs containing the WH2 motif behaved identically in this assay, and without the WH2 motif, no inhibition of polymerization was observed. The results from all constructs tested in this assay are summarized in the table in Figure 1(A). We conclude that the WH2 motif is necessary and, to the limits of this assay, sufficient for interaction with actin monomers and that the complex of MIM-B with actin monomer behaves qualitatively as sequestered actin, in a similar manner to other WH2 proteins or to profilin-actin complexes [27].

Since most of the MIM-B sequence is unique upstream of the WH2 motif, we used a yeast two-hybrid screen with 404-705MIM-B to search for MIM-B-interacting proteins. Screening a human brain library, we obtained two positive clones which both contained the PTP δ -CD. These were the only two positive clones identified in our screens with a human brain library (Clontech). We confirmed the interaction by retransformation of Y190 yeast with 404-705MIM-B and 404-759MIM-B and a cDNA fragment encoding the cytoplasmic domain of RPTP δ (Figure 4A). We used Scar1 and p21-Arc (ARPC3) as a negative

Figure 3 MIM-B sequesters actin monomers

$3 \mu\text{M}$ actin [10% (w/w) pyrene-labelled] was mixed with buffer or proteins as indicated and polymerization was induced at time zero. Polymerization of actin was calculated as a percentage of the control (maximum 100%). Fluorescence increase was measured over time as described in the Methods section. (A) Actin with increasing concentrations of 664-759MIM-B. (B) Actin with increasing concentrations of 404-759MIM-B. (C) Actin with increasing concentrations of 404-705MIM-B.

Figure 3 For legend, see facing page

Figure 4 MIM-B binds to RPTP δ through a central region

(A) Yeast two-hybrid data showing growth of yeast on 3AT plates dependent on the interaction of the two binding partners. In each case, the clone listed first is in the vector pYTH9 and the clone listed second is in pACT-II. β -galactosidase assays were performed on all positive interactions (results not shown) to confirm the interaction. Top row, left to right: positive control p21-ARC and Scar1-pACTII [30], negative controls p21-Arc and RPTP δ -CD clones 1 and 2. Second row, left to right: negative control MIM-B and Scar1, MIM-B and RPTP δ -CD clone 1, MIM-B and RPTP δ -CD clone 2. Bottom row, left to right: negative control 404-705MIM-B and Scar1, 404-705MIM-B and RPTP δ -CD clone 1, 404-705MIM-B and RPTP δ -CD clone 2. (B) Pull-down data showing that RPTP δ binds to MIM-B in COS-7 cells. COS-7 cells expressing myc-tagged MIM-B or human α -actinin (as a negative control) were lysed and incubated with glutathione-agarose bound to recombinant GST-PTP δ -CD or glutathione beads (see Methods section). Bound and free fractions were separated and run on SDS/PAGE for Western blotting. The blot was probed with anti-myc to detect tagged MIM-B (lanes 1, 11, 12) or 404-759MIM-B (lanes 5, 6, 9, 10) or myc- α actinin (lanes 13, 14). Lane 1, pellet showing MIM-B bound to RPTP δ -CD beads. Lane 2, pellet showing no MIM-B bound to empty beads. Lane 3, pellet showing no α -actinin bound to RPTP δ -CD beads. Lane 4, pellet showing no α -actinin bound to empty beads. Lanes 5 and 6, pellet showing 404-759MIM-B bound to RPTP δ -CD beads. Lanes 7 and 8, pellet showing no 404-759MIM-B bound to empty beads. Lane 9, supernatant from pull-down shown in lane 5. Lane 10, supernatant from pull-down shown in lane 7. Lane 11, supernatant from pull-down shown in lane 1. Lane 12, supernatant from pull-down shown in lane 2. Lane 13, supernatant from pull-down shown in lane 3. Lane 14, supernatant from pull-down shown in lane 4.

control for interaction with MIM-B and RPTP δ respectively (Figure 4A).

The yeast two-hybrid interaction of MIM-B with RPTP δ -CD was confirmed using recombinant RPTP δ -CD expressed in *E. coli* and fused to GST and various constructs of MIM-B expressed in COS-7 cells. Beads containing RPTP δ -CD, but not GST alone, bound to full-length MIM-B and to fragments of MIM-B containing amino acids 404–705 (Figure 4B). While we attempted to study the interaction between endogenous MIM-B and RPTP δ -

CD, we were unable to produce antisera against MIM-B which recognized the endogenous protein (results not shown), and although we obtained an antibody to RPTP δ -CD (kindly provided by Michel Streuli), we were not able to detect endogenous RPTP δ with this reagent (results not shown). It may be that these proteins are expressed at low levels, and/or that MIM-B is not particularly antigenic in rabbits, but so far, attempts with soluble protein, inclusion bodies and peptides have failed to produce a good quality antibody. A summary of all fragments of MIM-B which gave a positive interaction with PTP δ is shown in the table in Figure 1(A).

To determine whether MIM-B might be involved in actin cytoskeletal re-organization in cells and to investigate its cellular localization, we expressed myc-tagged full-length MIM-B and fragments encoding the N- and C-terminal portions of the protein. Full-length MIM-B induced actin-rich protrusions resembling surface ruffles and filopodia (Figure 5, panels A, A' and A''). Cells expressing MIM-B also lost stress fibres. This phenotype was observed in more than 70% of the cells ($n = 50$ cells). We observed the Arp2/3 complex co-localizing in the surface protrusions and ruffles, indicating that the Arp2/3 complex is likely to be involved in the actin assembly via MIM-B expression (Figure 5, panels B, B' and B''). Expression of the N-terminal portion of MIM-B (1-520MIM-B) did not affect the actin cytoskeleton detectably (Figure 5, panels C, C' and C'') but this construct localized to cytoplasmic vesicles (arrows on Figure 5C; more than 50% of the cells contained several vesicular structures; $n = 50$ cells). In contrast, the C-terminal portion of MIM-B (404-759MIM-B), containing the WH2 motif, showed a cytoplasmic localization and did not detectably affect the actin cytoskeleton (Figure 5, panels D, E, D', E' and D'', E''; $n = 50$ cells). We obtained similar results with both COS-7 cells and NIH3T3 cells for all constructs, and representative experiments are shown.

DISCUSSION

We have found that human MIM-B is an actin-monomer-binding protein that sequesters actin monomers *in vitro* in a similar manner to other WH2-containing proteins. MIM-B is conserved in mouse, *C. elegans* and *Drosophila*, and shows weak homology to BAIP1/IRSp53 proteins in the N-terminal portion. IRSp53 was originally identified as a substrate for the insulin receptor tyrosine kinase, so the homology between these proteins may reflect interaction sites for tyrosine kinases. Also, this is the region of MIM-B which localizes to membranes when expressed with a myc-tag, so it may be important for targeting to membranes. No other proteins were found to have significant homology to MIM-B, except at the region of the WH2 motif. Mattilla et al. [25], also recently characterized the mouse MIM homologue and found it to be an actin-binding protein that is primarily expressed in developing brain tissue and myoblasts.

MIM-B binds to actin monomers with a micromolar affinity in bead pelleting assays, which is also similar to the affinities reported for WASP-family proteins, or actobindin for actin monomers [21,22]. The WH2 motif is required for this actin-binding activity. The WH2 motif of MIM-B appears to have a somewhat lower affinity for actin monomers than that of the recently identified mouse MIM ($0.5 \mu\text{M}$ for human versus $0.06 \mu\text{M}$ for mouse, see [25]). We do not know whether this is due to a true difference between the mouse and human proteins or to the experimental methods employed in the two laboratories, but K_d values in the low micromolar range are similar to the literature values for other WH2-containing proteins. Differences reported may possibly be due to the alternatively spliced MIM variant

Figure 5 Expression of MIM-B constructs in cells disrupts the actin cytoskeleton

(A) Full-length myc-tagged MIM-B expressed in NIH3T3 cells and stained with anti-myc. (A') F-actin staining with phalloidin in MIM-B overexpressing cells. (A'') Overlay of MIM-B (green) and actin (red). (B) Expression of full-length myc-tagged MIM-B in COS-7 cells stained with anti-myc. (B') The same cell stained with anti-p34-Arc (ARPC2). (B'') Overlay of anti-myc (green) and anti-p34-Arc (red) staining. (C) Expression of myc-tagged 1-520MIM-B in NIH3T3 cells and staining with anti-myc. Red arrow points to vesicular staining. (C') F-actin staining with phalloidin in same cells. (C'') Overlay with 1-520MIM-B (in green) and F-actin (in red). (D, E) Myc-tagged 404-759MIM-B expressed in NIH3T3 cells and stained with anti-myc. (D', E') F-actin stained with phalloidin in same cells. (D'', E'') Overlay with 404-759MIM-B (in green) and F-actin (shown in red). Scale bars in (B, D, E) represent 5 μ m. Scale bars in (A, C) represent 20 μ m.

used for the mouse studies [25]. The minimal MIM-B fragment that bound to G-actin in the pulldown assay was 565-759MIM-B, while the much smaller 706-759MIM-B still showed actin monomer sequestering activity in the kinetics assays. This may be due to the GST-fusion interfering with actin monomer binding in the pulldown assays with short fragments, or it may be that the shorter fragments have a sufficiently low affinity for actin monomer that they are not retained during the washes of the pulldown experiment. In both experimental systems, the WH2 motif was necessary for actin binding and in the kinetic experiments, it was sufficient for monomer sequestration.

While we do not yet know the function of the actin-binding activity of MIM-B, we can speculate that, like other WH2 proteins, MIM-B has at least three possible activities. First, it may be an actin-monomer-binding protein that regulates filament assembly, similar to thymosin/actobindin or cibolout. Thymosin and actobindin are thought to act as a simple actin monomer sequestering proteins, while cibolout enhances the assembly of actin monomers on the barbed ends of filaments [26,28,29]. Secondly, MIM-B may be a polarity-determining protein, similar to verprolin [7,8]. Finally, MIM-B may act as a scaffold protein to activate actin assembly similar to WASP-family proteins [2]. Given the interaction of MIM-B with RPTP δ and the induction of actin-rich protrusions upon overexpression of MIM-B, we favour the idea that it may be involved in polarity or actin assembly.

The mechanism of induction of surface protrusions by MIM-B is unknown, but our immunolocalization studies suggest that it involves the Arp2/3 complex and actin. We speculate that MIM-B may be a part of a larger complex that induces actin assembly at the plasma membrane, similar to verprolin or WIP, which co-operate with WASP-family proteins to recruit Arp2/3-complex-mediated actin assembly complexes. Further investigation into the regulation of MIM-B by small GTPases and possible interactions with WASP-family proteins or the Arp2/3 complex may reveal whether there is any connection.

The loss of actin stress fibres upon overexpression of MIM-B may be due to a re-direction of actin into surface protrusions, a loss of focal adhesion complexes, an inactivation of myosin-II or even a change in the composition/activation state of cell surface receptors. Fragments of WASP-family proteins when over-expressed cause an eventual loss of actin stress fibres, as they redirect much of the cellular F-actin into a diffuse cytoplasmic localization [30]. Alternatively, the interaction of MIM-B with RPTP δ may change the tyrosine phosphorylation state of focal adhesion proteins and thus regulate the disassembly of integrin complexes, which would subsequently lead to stress fibre disassembly. If MIM-B is involved in actin assembly at the plasma membrane, it may regulate the endocytosis or recycling of surface receptors, thus changing the activation state of integrin complexes via alterations in membrane trafficking. The membrane localization of the N-terminal portion of MIM-B suggests that this possibility warrants further investigation.

Taken altogether, our study identifies human MIM-B, a potential suppressor of metastasis, as an actin and RPTP δ -binding protein that may link signalling and the cytoskeleton. Our data point to the idea that the mechanism of metastasis suppression by MIM may be via actin re-organization and/or changes in cell adhesion or tyrosine phosphorylation upon loss of MIM. Mouse MIM was identified by another group [25] to have similar actin-binding activities and to be widely distributed in mouse tissues, but highly expressed in developing neurons and myoblasts, suggesting a role in cell polarity. Future studies with MIM proteins will hopefully reveal how these newly identified WH2-containing proteins control actin cytoskeletal assembly and how they may prevent the metastasis of cancer cells.

We thank Michel Streuli for the gift of the RPTP δ expression construct and antibody. We thank Guillaume Bompard for helpful advice. We thank the referees of our manuscript for their excellent advice on the naming of MIM-B and its likelihood of being a splice variant of MIM. L.M. and J.W. are supported by an MRC Senior Fellowship to L.M. and S.J.S. is supported by a BBSRC studentship.

REFERENCES

- Higgs, H. N. and Pollard, T. D. (2001) Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem.* **70**, 649–676.
- Cory, G. O., Garg, R., Cramer, R. and Ridley, A. J. (2002) Phosphorylation of tyrosine 291 enhances the ability of WASp to stimulate actin polymerization and filopodium formation. Wiskott-Aldrich Syndrome protein. *J. Biol. Chem.* **277**, 45115–45121.
- Scaplehorn, N., Holmstrom, A., Moreau, V., Frischknecht, F., Reckmann, I. and Way, M. (2002) Grb2 and Nck act cooperatively to promote actin-based motility of vaccinia virus. *Curr. Biol.* **12**, 740–745.
- Carlier, M. F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P. and Pantaloni, D. (2000) GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASP) with actin-related protein (ARP2/3) complex. *J. Biol. Chem.* **275**, 21946–21952.
- Paunola, E., Mattila, P. K. and Lappalainen, P. (2002) WH2 domain: a small, versatile adapter for actin monomers. *FEBS Lett.* **513**, 92–97.
- Naqvi, S. N., Zahn, R., Mitchell, D. A., Stevenson, B. J. and Munn, A. L. (1998) The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast. *Curr. Biol.* **8**, 959–962.
- Donnelly, S. F., Pocklington, M. J., Pallotta, D. and Orr, E. (1993) A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **10**, 585–596.
- Vaduvu, G., Martin, N. C. and Hopper, A. K. (1997) Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton. *J. Cell Biol.* **139**, 1821–1833.
- Kerkhoff, E., Simpson, J. C., Leberfinger, C. B., Otto, I. M., Doerks, T., Bork, P., Rapp, U. R., Raabe, T. and Pepperkok, R. (2001) The Spir actin organizers are involved in vesicle transport processes. *Curr. Biol.* **11**, 1963–1968.
- Manseau, L. J. and Schupbach, T. (1989) Cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Dev.* **3**, 1437–1452.
- Wellington, A., Emmons, S., James, B., Calley, J., Grover, M., Tolias, P. and Manseau, L. (1999) Spire contains actin binding domains and is related to ascidian posterior end mark-5. *Development* **126**, 5267–5274.
- Lee, Y. G., Macoska, J. A., Korenchuk, S. and Pienta, K. J. (2002) MIM, a potential metastasis suppressor gene in bladder cancer. *Neoplasia* **4**, 291–294.
- Pulido, R., Krueger, N. X., Serra-Pages, C., Saito, H. and Streuli, M. (1995) Molecular characterization of the human transmembrane protein-tyrosine phosphatase δ . Evidence for tissue-specific expression of alternative human transmembrane protein-tyrosine phosphatase delta isoforms. *J. Biol. Chem.* **270**, 6722–6728.
- Lammers, R., Lerch, M. M. and Ullrich, A. (2000) The carboxyl-terminal tyrosine residue of protein-tyrosine phosphatase α mediates association with focal adhesion plaques. *J. Biol. Chem.* **275**, 3391–3396.
- Adamsky, K., Schilling, J., Garwood, J., Faissner, A. and Peles, E. (2001) Glial tumor cell adhesion is mediated by binding of the FNIII domain of receptor protein tyrosine phosphatase β (RPTP β) to tenascin C. *Oncogene* **20**, 609–618.
- Bixby, J. L. (2000) Receptor tyrosine phosphatases in axon growth and guidance. *Neuroreport* **11**, R5–R10.
- Wang, J. and Bixby, J. L. (1999) Receptor tyrosine phosphatase-delta is a homophilic, neurite-promoting cell adhesion molecule for CNS neurons. *Mol. Cell. Neurosci.* **14**, 370–384.
- Wills, Z., Bateman, J., Korey, C. A., Comer, A. and Van Vactor, D. (1999) The tyrosine kinase Abl and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. *Neuron* **22**, 301–312.
- Bateman, J., Reddy, R. S., Saito, H. and Van Vactor, D. (2001) The receptor tyrosine phosphatase Dlar and integrins organize actin filaments in the *Drosophila* follicular epithelium. *Curr. Biol.* **11**, 1317–1327.
- Bateman, J., Shu, H. and Van Vactor, D. (2000) The guanine nucleotide exchange factor trio mediates axonal development in the *Drosophila* embryo. *Neuron* **26**, 93–106.
- Spudich, J. A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866–4871.

- 22 Pollard, T. D. and Cooper, J. A. (1984) Quantitative analysis of the effect of Acanthamoeba profilin on actin filament nucleation and elongation. *Biochemistry* **23**, 6631–6641
- 23 Mullins, R. D. and Machesky, L. M. (2000) Actin assembly mediated by Arp2/3 complex and WASP family proteins. *Methods Enzymol.* **325**, 214–237
- 24 Aspenstrom, P. and Olson, M. F. (1995) Yeast two-hybrid system to detect protein-protein interactions with Rho GTPases. *Methods Enzymol.* **256**, 228–241
- 24a Machesky, L. M. and Hall, A. (1997) Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. *J. Cell Biol.* **138**, 913–926
- 25 Mattila, P. K., Salminen, M., Yamashiro, T. and Lappalainen, P. (2003) Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain. *J. Biol. Chem.* **278**, 8452–8459
- 26 Bubb, M. R., Lewis, M. S. and Korn, E. D. (1991) The interaction of monomeric actin with two binding sites on Acanthamoeba actobindin. *J. Biol. Chem.* **266**, 3820–3826
- 27 Higgs, H. N., Blanchoin, L. and Pollard, T. D. (1999) Influence of the C terminus of Wiskott-Aldrich syndrome protein (WASP) and the Arp2/3 complex on actin polymerization. *Biochemistry* **38**, 15212–15222
- 28 Safer, D., Elzinga, M. and Nachmias, V. T. (1991) Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. *J. Biol. Chem.* **266**, 4029–4032
- 29 Boquet, I., Boujemaa, R., Carlier, M. F. and Preat, T. (2000) Ciboulot regulates actin assembly during Drosophila brain metamorphosis. *Cell* **102**, 797–808
- 30 Machesky, L. M. and Insall, R. H. (1998) Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8**, 1347–1356

Received 18 December 2002/30 January 2003; accepted 5 February 2003

Published as BJ Immediate Publication 5 February 2003, DOI 10.1042/BJ20021962