Dimerization is necessary for MIM-mediated membrane deformation and endocytosis

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MIM [missing in metastasis; also called MTSS1 (metastasis suppressor 1)] is an intracellular protein that binds to actin and cortactin and has an intrinsic capacity to sense and facilitate the formation of protruded membranous curvatures implicated in cellular polarization, mobilization and endocytosis. The N-terminal 250 amino acids of MIM undergo homodimerization and form a structural module with the characteristic of an I-BAR [inverse BAR (Bin/amphiphysin/Rvs)] domain. To discern the role of the dimeric configuration in the function of MIM, we designed several peptides able to interfere with MIM dimerization in a manner dependent upon their lengths. Overexpression of one of the peptides effectively abolished MIM-mediated membrane protrusions and transferrin uptake. However, a peptide with a high potency inhibiting MIM dimerization failed to affect its binding to actin and cortactin. Thus the results of the present study indicate that the dimeric configuration is essential for MIM-mediated membrane remodelling and serves as a proper target to develop antagonists specifically against an I-BAR-domain-containing protein.

Key words: actin and cortactin, dimerization, inverse Bin/amphiphysin/Rvs (I-BAR), missing in metastasis (MIM).

INTRODUCTION

Cellular activities, including migration, intracellular trafficking and endocytosis, require extensive dynamic membranous curvatures that are often ultimately manifested as microprotrusions, invaginations, vesicles and tubules. Accumulated evidence has indicated that initiation and maintenance of these curvatures in mammalian cells is accomplished by the reversible assembly of a series of membrane-sculpting or -deforming proteins. Many of these proteins contain a common structural domain that was initially recognized in Bin, amphiphysin and Rvs proteins and thus constitute a superfamily known as BAR (Bin/amphiphysin/Rvs) [1–3]. On the basis of the intrinsic curvature of the BAR domain, members of the BAR family can be further divided into several subfamilies, including classical BAR, N-BAR (N-terminal amphipathic helix-BAR), BAR-PH (BAR-pleckstrin homology), PX-BAR (PhoX-BAR), F-BAR (Fes/CIP4 homology-BAR) and I-BAR (inverse BAR) [4]. Although most BAR domains have a positively charged concave surface, an I-BAR domain displays a convex exterior [5,6]. Hence a protein containing an I-BAR domain tends to sense the formation of membrane protrusions rather than invaginations, which are often implemented by other BAR subfamilies. Besides MIM (missing in metastasis), mammalian genomes contain four additional I-BAR genes, encoding IRSP53 (insulin receptor substrate protein of 53 kDa), ABBA (actin-bundling protein with BAIBAP2 homology), IRTKS (insulin receptor tyrosine kinase substrate) and PINKBAR (planar intestinal- and kidney-specific BAR) respectively [7,8]. Among them, MIM has drawn particular attention because of its putative role in tumour progression and frequent underexpression in a set of metastatic cells [9–11]. MIM displays typical I-BAR properties when analysed in vitro and in vivo. Overexpression of MIM leads to an increase in the formation of filopodia-like micropseudopodial protrusions of a variety of cells [12,13]. On the other hand, depletion of MIM genes in animals impairs cell polarization, chemotactic responses to chemokines, endocytosis of ligand-occupied receptors and cell–cell interactions in certain tissues [14–16]. Although the existing data support a critical role of MIM in membrane deformation, the structural basis for the function of MIM remains unclear.

The ability to induce dynamic membrane remodelling by MIM is presumably attributed to the property of its I-BAR domain. Indeed, it has been reported that the MIM I-BAR binds to PtdIns(4,5)P₂-enriched membranes [17], interacts with the small GTPase Rac [18] and induces membranous tubules in vitro [17]. However, MIM is distinct from IRSP53 and its closely related I-BAR proteins by containing a SRD (serine-rich domain), a PRD (proline-rich domain) and a C-terminal WH2 (Wiskott–Aldrich syndrome homology region 2) motif (Figure 1A). Although the biochemical function of these domains remains elusive, they are associated with at least three intracellular proteins: monomeric actin [12], cortactin [13] and Daam1 (dishevelled-associated activator of morphogenesis 1) [19]. Significantly, all of these proteins are well-characterized components of the cortical microfilament [20,21], a major cytoskeletal force that drives membrane dynamics. Therefore MIM could also deform membranes through a process coupling with microfilament-associated proteins.

To dissect the specific function of the I-BAR of MIM, it would be necessary to modulate the I-BAR without dramatic

Abbreviations used: BAR, Bin/amphiphysin/Rvs; Bio-Tfn, biotin-labelled transferrin; Daam1, dishevelled-associated activator of morphogenesis 1; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GST, glutathione transferase; HEK, human embryonic kidney; I-BAR, inverse BAR; IRSP53, insulin receptor substrate protein of 53 kDa; LB, Lennox broth; MIM, missing in metastasis; PRD, proline-rich domain; SH3, Src homology 3; SRD, serine-rich domain; WH2, Wiskott–Aldrich syndrome homology region 2.

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disturbance of other motifs. Since the MIM I-BAR undergoes a homodimerization, which builds up a zeppelin-like module for membrane binding [5,22], we thought that it may be useful to develop a specific I-BAR antagonist by targeting the dimerization. In the present study, we designed several peptides with inhibitory activities for MIM dimerization and provided evidence for the necessity of I-BAR dimerization in MIM-mediated endocytosis and membrane protrusions. However, a peptide with a strong activity antagonizing MIM I-BAR dimerization did not affect the association of MIM with cortactin and actin, demonstrating a distinct role of the I-BAR domain in membrane remodelling.

EXPERIMENTAL

Chemicals and antibodies

All chemicals unless otherwise indicated were purchased from Sigma–Aldrich. Protein A– and Protein G–agarose were from Santa Cruz Biotechnology. Glutathione–Sepharose 4B was from Qiagen. and GeneJammer was from Stratagene. Anti-GFP (green fluorescent protein) antibody was raised against recombinant His6–GFP protein as described previously [23]. An anti-Myc monoclonal antibody (9E10) was from BD Biosciences. An anti-cortactin protein as described previously [23]. An anti-Myc monoclonal antibody (4F11) was from Millipore.

Plasmids

MIM–GFP, MIM-I-BAR–GFP, FLAG–MIM, GST (glutathione transferase)–MIM, GST–MIM-I-BAR, His6–MIM, and MIM-I-BAR–Myc were prepared as described previously [24]. Plasmids encoding Myc–, GFP– and GST-tagged MIM-S1 or MIM-S2 peptides were prepared by ligation of PCR-generated DNA fragments into vectors pcDNA3.1A (Invitrogen), pEGFP-N1 (Clontech) and PGEX4T-2 (Amersham) respectively. The primers used in PCR were as follows (5′-primer and 3′-primer respectively): pMIM-S1-Myc, 5′-CATGTTAGATCCATGACCGCTCAGTG-3′ and 5′-CATGTTCTGACCTAGGGATTGTCAGTG3′; pMIM-S2-Myc, 5′-CATGTTAGATCCATGACCGCTCAGTG-3′ and 5′-CATGTTCTGACCTAGGGATTGTCAGTG3′; pGST-MIM-S1, 5′-CATGTTAGATCCATGACCGCTCAGTG-3′ and 5′-CATGTTCTGACCTAGGGATTGTCAGTG3′; pGST-MIM-S2, 5′-CATGTTAGATCCATGACCGCTCAGTG-3′ and 5′-CATGTTCTGACCTAGGGATTGTCAGTG3′; pMIM-S1-GFP, 5′-CATGTTACTCGAGATGCGCAAGGGCA-3′ and 5′-CAGTTTAGATCCCGGAGTCTCAGGGTCG-3′; and pMIM-S2-GFP, 5′-CAGTTACTCGAGATGCGCAAGGGCA-3′ and 5′-CAGTTAGATCCCGGAGTCTCAGGGTCG-3′.

Recombinant proteins and peptides

His6– or GST-tagged proteins, including His6–MIM, GST–MIM-S1 and -S2, and GST–MIM-I-BAR, were prepared as described previously [13]. The brief procedure to purify GST-tagged proteins was as follows. DH5α bacterial cells were transformed by plasmids, and the transformed cells were selected on the basis of resistance to ampicillin. A single colony of the transformed cells was added to 3 ml of LB (Lennox broth) (Invitrogen) containing 30 μg/ml ampicillin and incubated at 37°C for 8–16 h at a shaking speed of 200 rev/min. The culture was further inoculated into 100 ml of pre-warmed LB with 50 μg/ml ampicillin for 1–2 h. When the attenuation at 600 nm reached to 0.3 or 0.4, 0.5 mM isopropl-β-D-thiogalactopyranoside was added to the culture. After an additional 90 min of incubation, cells were harvested in PBS, sonicated with a Sonics-Cell VC505 (Sonics & Materials) using a micro-tip at a setting of 30% amplitude (six pulses each of 10 s duration), and centrifuged at 16,000 g and 4°C for 20 min. The supernatants of lysed cells were incubated with glutathione–Sepharose 4B for 2 h at 4°C followed by three washes with PBS and were stored in PBS containing 0.02% NaN3. If bead-free proteins were needed, GST-tagged proteins were eluted with 10 mM GSH in 50 mM Tris/HCl, pH 8.0, and dialysed against PBS using a Centricon YM-30 filter (Millipore). Protein concentrations were determined by comparing with BSA in either a Protein Assay (Bio-Rad Laboratories) or by SDS/PAGE followed by Coomassie Blue (Bio-Rad Laboratories) staining. The synthetic peptides MIM-S3, -S4, -S5 and -S6 were commercially prepared by GenScript.

Cell culture

HEK (human embryonic kidney)-293T cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) (Lonza) supplemented with 10% (v/v) fetal bovine serum (HyClone, Thermo Scientific), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). For DNA-mediated transfection, 1.5×10⁶ cells were seeded in a 100-mm-diameter dish in growth medium
(DMEM plus 10% fetal bovine serum) without antibiotics and incubated at 37°C and 5% CO2. After 12 h, transfection complexes were prepared by mixing 10 μg of DNA, 300 μl of DMEM and 60 μl of SuperFect or GeneJammer, and incubated at room temperature (25°C) for 5 to 10 min followed by addition into 3 ml of regular growth medium [DMEM plus 10% (v/v) fetal bovine serum]. The mixture was then used to grow cells to be transfected at 37°C for 2–3 h followed by transfer into pre-warmed regular growth medium. After 48 h, the transfected cells were harvested for subsequent analysis.

**Immunoprecipitation and Western blot analysis**

Cell lysates were clarified by centrifugation at 16 000 g for 5 min. A 25 μl portion of a 50% (v/v) slurry of Protein A– or Protein G–Sepharose and 5 μg of polyclonal anti-GFP antibody were added to the clarified lysates. In some experiments, 1 μg of anti-Myc monoclonal antibody or anti-FLAG monoclonal antibody was used in the reactions. The mixtures were then incubated for 2 h at 4°C on a rotation wheel, and briefly spun down. A portion (10 μl) of the supernatants or the pellets (after three washes with PBS) were fractionated by SDS/PAGE, transferred on to a nitrocellulose membrane and subjected to Western blotting with either anti-GFP or anti-Myc followed by horseradish-peroxidase-conjugated secondary antibody in 5% (w/v) non-fat milk powder. The antibody-reactive substances on the membrane were detected by chemiluminescence and digitally visualized by Kodak ImageStation 2000 using Kodak 1D 3.6 software.

**Dimerization analysis**

HEK-293T cells were co-transfected with pMIM-GFP and pFLAG-MIM. After 1 or 2 days of transfection, cell lysates were prepared and then subjected to immunoprecipitation using an anti-FLAG antibody. Unbound MIM–GFP in the supernatants was detected by Western blotting with an anti-GFP antibody. In some experiments, cells were co-transfected with pMIM-Myc and pFLAG-MIM. In these experiments, anti-Myc and anti-FLAG antibodies were used in immunoprecipitation and Western blotting respectively.

To analyse recombinant MIM proteins, GST–MIM-I-BAR at different concentrations was incubated with 25 nM His6–MIM for 2 h at 37°C and an additional 12 h at 4°C in 500 μl of PBS supplemented with 20 μg/ml BSA, 10 mM PMSF and one tablet of Roche protease inhibitor per ml. After incubation, 40 μl of 50% (v/v) glutathione–Sepharose was added to the samples and incubation was continued for 30 min at 4°C. The complex of dimerized MIM proteins was precipitated, separated by 10% (v/v) SDS/PAGE and detected by Western blotting using a polyclonal anti-MIM antibody.

**Inhibition of MIM dimerization**

Cell lysates derived from HEK-293T cells co-transfected with MIM–GFP (or FLAG–MIM) and MIM–Myc were incubated with GST–MIM-S1 or synthetic peptides at different concentrations at 4°C for 2 h. Dimerization of MIM–GFP (or FLAG–MIM) with MIM–Myc in the reactions was measured as described above. The intensity of acquired digital bands was quantified by ImageJ software and normalized to percentages as compared with that with the lysate prior to immunoprecipitation and that with the lysate in the absence of antagonists. The normalized values were further plotted as a function of MIM antagonists and used to deduce IC50 values using Prism 5 software.

**Endocytosis assay**

Endocytosis was analysed as described previously [23]. Briefly, cells were seeded in 12-well plates at a density of 5 × 10^5 cells per well and cultured overnight in DMEM supplemented with 10% (v/v) fetal bovine serum. Prior to analysis the cells were starved in DMEM supplemented with 1% BSA and 20 mM Hepes at 37°C for 30 min. Bio-Tfn (biotin-labelled transferrin) was added at 10 μg/ml and incubated for 40 min on ice. To initiate endocytosis, cells were transferred to a 37°C incubator and incubated for 5 min. Endocytosis was terminated by placing the cells back on ice. The treated cells were lysed, and the total cell lysates were then transferred to a 96-well ELISA plate (Thermo) that had been pre-coated with transferrin antibody and incubated overnight at 4°C. The plate was then washed and treated with streptavidin–horseradish peroxidase followed by BM Blue substrate (Roche). Absorption at 450 nm was determined by a microplate reader (Thermomax).

**Immunofluorescence**

HEK-293T cells were co-transfected with pMIM-GFP and pMIM-S1-Myc. After 24 h of transfection, the cells were trypsinized and seeded on to six-well plates containing a sterilized fibronectin-coated coverslip and cultured in normal growth medium overnight. The cells were fixed by 4% (w/v) paraformaldehyde for 20 min and then permeabilized by 0.5% Triton X-100 in PBS for 5 min at room temperature. After three washes with PBS, cells were incubated in PBS containing 5% (v/v) BSA for 30 min and treated with primary antibody for 1 h followed by fluorophore-conjugated secondary antibody for an additional 1 h in PBS plus 5% (v/v) BSA. The stained cells were mounted on to slides, sealed with nail polish and viewed under a fluorescence microscope (Nikon TE2000-U) using a ×60 oil-immersion objective lens with numerical aperture of 1.40. Digital images were captured by a Nikon DXM1200 camera.

**RESULTS AND DISCUSSION**

To analyse MIM dimerization, we prepared two constructs in which MIM was tagged by GFP and Myc epitopes at the C-terminus respectively and co-transfected them into HEK-293T cells. Immunoprecipitation with an anti-Myc antibody followed by Western blotting using an anti-GFP antibody demonstrated a stable association between MIM–Myc and MIM–GFP (Figure 1B). We also analysed cells co-expressing MIM–Myc and FLAG–MIM, and verified the presence of a dynamic intramolecular interaction of MIM proteins tagged by different epitopes in cells (Figure 1C).

Crystallization studies have revealed that the N-terminal 250 amino acids of MIM are organized into three helices (Figure 2A, top panel; each helix is depicted by two tubes). In the twisted helices, the interface for dimerization lies within two areas [5]: most of the first helix and the C-terminal part of the third helix. We reasoned that a peptide corresponding to each interface area may have an ability to disrupt MIM dimerization, thereby acting as an effective MIM antagonist. Thus we designed two peptides, MIM-S1 and MIM-S2, which correspond to each interface area as described above. The intensity of acquired digital bands was quantified by ImageJ software and normalized to percentages as compared with that with the lysate prior to immunoprecipitation and that with the lysate in the absence of antagonists. The normalized values were further plotted as a function of MIM antagonists and used to deduce IC50 values using Prism 5 software.

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The structures and sequences of MIM-S1 and MIM-S2. Top panel: a single MIM-I-BAR subunit is shown in a tube format based on Lee et al. [5]. The residues corresponding to MIM-S1 and MIM-S2 are represented as dark grey and light grey balls respectively. Middle panel: two MIM-I-BAR dimers are depicted in space-filling format. The areas in the lightest colour represent the residues for MIM-S1 and MIM-S2 respectively. Bottom panel: amino acid sequences for MIM-S1 and MIM-S2. (B) Recombinant GST, GST–MIM-S1 and GST–MIM-S2 proteins were analysed by SDS/PAGE followed by Coomassie Blue staining. Note that GST–MIM-S2 displayed a significant degradation. (C) Lysates derived from HEK-293T cells expressing either MIM–Myc (upper panel) or MIM-I-BAR–Myc (lower panel) were subject to pull-down with GST, GST–MIM-S1 and GST–MIM-S2 respectively. The presence of MIM–Myc or MIM-I-BAR–Myc in the precipitates were detected by Western blotting using an anti-Myc antibody. (D) Lysates of cells co-transfected with different combinations of FLAG–MIM, MIM-S1–GFP and MIM-S2–GFP were immunoprecipitated by an anti-FLAG antibody, and the immunopellets were analysed by Western blotting using an anti-GFP antibody. Expression of MIM-S1–GFP and MIM-S2–GFP in total lysates is shown in the lower panel. TCL, total cell lysates.

conditions (Figure 2C). To verify the interaction within cells, we also prepared MIM-S1 and MIM-S2 tagged by GFP and co-transfected them individually with FLAG–MIM into HEK-293T cells. As shown in Figure 2(D), MIM-S1–GFP, but not MIM-S2–GFP, co-precipitated readily with FLAG–MIM. The poorer ability of MIM-S2 to interact with MIM could be due to a smaller interface area it corresponds to and its lower stability as evidenced by significant degradation expressed in either bacterial (Figure 2B) or HEK-293T cells (Figure 2D and results not shown). Since we could not consistently obtain high expression levels of intact MIM-S2, we used MIM-S1 and its derivatives for the rest of the present study.

To quantify the interaction between MIM-S1 and MIM, GST–MIM-S1 at different concentrations was used to precipitate the lysates of cells expressing either MIM–Myc or MIM-I-BAR–Myc. The MIM proteins remaining in the supernatants after precipitations were measured by Western blotting and used as the index for unbound MIM proteins. This analysis estimated that MIM-S1 has a $K_d$ value of 110 nM for MIM–Myc and 65 nM for MIM-I-BAR–Myc (Figure 3A). The apparently higher affinity for MIM-I-BAR–Myc could be due to other domains in full-length MIM (Figure 1A), which may restrict sterically the access to the MIM antagonist. Stable and direct association of MIM-S1 with MIM was further confirmed using purified GST–MIM-S1 and His$_6$–MIM (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460469add.htm).

We next analysed whether the MIM-S1 peptide would be able to disrupt MIM dimerization. Thus the lysate of cells co-expressing FLAG–MIM and MIM–GFP was incubated with GST–MIM-S1 at different concentrations followed by immunoprecipitation of MIM–GFP. Unpaired FLAG–MIM remaining in the supernatant was estimated by Western blot analysis. As shown in Figure 4(A), the presence of GST–MIM-S1 increased markedly the amount of unpaired FLAG–MIM, indicative of disruption in MIM dimerization. Quantification estimated an IC$_{50}$ value of 213 nM for GST–MIM-S1 (Figure 4A). To rule out the possibility that the GST in GST–MIM-S1 might have contributed to the inhibition, we prepared a series of synthetic peptides corresponding to the C-terminal part of MIM-S1 (Supplementary Table S1 at http://www.BiochemJ.org/bj/446/bj4460469add.htm) and analysed their effect on MIM dimerization. One peptide, MIM-S3, which has 33 amino acids, was able to inhibit effectively MIM dimerization with an IC$_{50}$ value of 140 nM (Figure 4B). The peptides ranging from 6 to 20 amino acids within the same region showed a diminished inhibitory activity as their length was reduced (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460469add.htm), suggesting a minimal number of residues necessary for effective competition with MIM dimerization. To verify that the inhibition was not due to unknown cellular factors that might have been co-purified during immunoprecipitation, we also analysed MIM dimerization using purified recombinant GST–MIM-I-BAR and His$_6$–MIM proteins. Under this condition, MIM-S3 peptide displayed a strong inhibitory activity with an IC$_{50}$ value of approximately 41 nM (Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460469add.htm).

We recently reported that overexpression of MIM–GFP increased the endocytosis of transferrin [23], and we were
Inhibition of MIM dimerization

Figure 3  Quantification of the affinity of GST–MIM-S1 for MIM

The lysates of cells expressing MIM–Myc (A) or MIM-I-BAR–Myc (B) were incubated with GST–MIM-S1 at the concentrations indicated. The GST–MIM-S1 binding proteins were isolated by pull-down. The amounts of unbound MIM–Myc or MIM-I-BAR–Myc proteins in the supernatants after precipitation were measured by Western blotting using an anti-Myc antibody. As the loading control, β-actin in the supernatants was also measured. Data were analysed by Prism software, which predicted $K_d$ values of 110 nM and 65 nM for MIM–Myc and MIM-I-BAR–Myc respectively.

Figure 4  Analysis of the activity of MIM-S1 and MIM-S3 to antagonize MIM dimerization

(A) Lysates of cells expressing FLAG–MIM and MIM–GFP were incubated with GST–MIM-S1 for 2 h and subjected to immunoprecipitation with an anti-GFP antibody. Unbound FLAG–MIM in the supernatants were determined by Western blotting using an anti-FLAG antibody, normalized and plotted as a function of GST–MIM-S1. IC$_{50}$ values were calculated using Prism software. As a control, aliquots of cell lysates were also subjected to Western blotting using an anti-β-actin antibody. (B) The lysates of cells expressing MIM–Myc and MIM–GFP were incubated with MIM-S3 peptide at various concentrations as indicated and then subjected to immunoprecipitation using an anti-Myc antibody. Unbound MIM–GFP was detected by Western blotting using an anti-GFP antibody and used to estimate IC$_{50}$ values.

interested in whether MIM-S1 has any influence on MIM-mediated transferrin uptake. Consistent with our previous finding with NIH 3T3 cells [23], HEK-293T cells expressing MIM-GFP alone displayed a marked increase in the uptake of transferrin by 30% (Figure 5A). However, the increase was diminished in cells co-expressing MIM-S1–Myc and MIM–GFP. The possible toxicity of MIM-S1–Myc to endocytosis was unlikely because overexpression of MIM-S1–Myc alone in HEK-293T cells, where little endogenous MIM was expressed (results not shown), did not affect the internalization of transferrin. Thus the inhibition manifested by MIM-S1–Myc was specific for the MIM-mediated endocytosis. We also analysed the effect on membrane deformations mediated by overexpressing MIM–GFP, which provoked the formation of MIM-characteristic filopodia-like protrusions (Figure 5B, arrowhead). However, co-expressing MIM–GFP and MIM-S1–Myc reduced the number of these protrusions significantly (Figure 5B, *) ($P < 0.0001$).

The critical role of dimerization in the function of other BAR-containing proteins has also been described previously. It has been reported recently that a mutation-mediated disruption of dimerization of endophilin, an N-BAR protein, impairs endocytosis and membrane shaping [25]. The report is essentially
expressing MIM–GFP alone. MIM-S1–Myc had approximately 70% fewer MIM-mediated membrane protrusions than those protrusions. Quantification (lower panel) showed that cells co-expressing MIM–GFP and anti-Myc antibodies. The stained cells were inspected by immunofluorescent microscopy. Cells were transiently transfected with MIM–GFP and MIM-S1–Myc, fixed and stained with 480 amino acids respectively (Figure 1A). In contrast, the SH3 domain of endophilin is only 41 amino acids apart from its BAR domain, suggesting that dimerization may only influence the partner that binds in the vicinity of the BAR domain. In this regard, it is noteworthy that there is an SRD motif in juxtaposition to the C-terminus of MIM’s I-BAR domain (Figure 1A), and that a peptide corresponding to the SRD is reportedly able to bind to Daam1, a formin-like protein that regulates actin polymerization [19]. In addition, IRSP53 and its related proteins PINKBAR and IRTKS contain an SH3 domain adjacent to the C-terminal part of their I-BAR domains. Although it would be interesting to determine whether dimerization could regulate Daam1 or any SH3-binding proteins in other I-BAR proteins, it has been well established that overexpression of an I-BAR domain itself is potent enough to induce membrane deformation [12,17,26]. Thus our finding supports the notion that the dimeric MIM-I-BAR is a functional rather than a regulatory motif, which may act in concert with other domains in recruiting membranous curvatures to the locations where actin filaments are actively assembled upon different signalling circuities. The peptides against MIM dimerization described in the present study would serve as useful tools to distinguish the membrane remodelling function of MIM from those linking to the actin dynamics, which may ultimately provide insight into the mechanics underlying membrane-related activities such as intracellular trafficking and cell migration.

Figure 5 MIM-S1 inhibits MIM-mediated endocytosis and membrane protrusions

(A) Cells co-expressing MIM–S1–Myc and MIM–GFP or cells expressing GFP only were treated with Bio-Tfn for 5 min. Uptake of Bio-Tfn was analysed as described in the Experimental section. Although Tfn uptake was markedly increased in cells expressing MIM–GFP, no significant increase was observed in cells expressing both MIM–GFP and MIM–S1–Myc. (B) HEK-293T cells were transiently transfected with MIM–GFP and MIM-S1–Myc, fixed and stained with anti-Myc antibodies. The stained cells were inspected by immunofluorescent microscopy. Original magnification, ×600. A cell showing expression of MIM–GFP only exhibited many filopodia-like long membrane protrusions as indicated by an arrowhead. A cell, indicated by *, expressing both MIM–GFP and MIM-S1–Myc failed to display these MIM-characteristic protrusions. Quantification (lower panel) showed that cells co-expressing MIM–GFP and MIM-S1–Myc had approximately 70% fewer MIM-mediated membrane protrusions than those expressing MIM–GFP alone.

consistent with the results of the present study that dimerization is essential for I-BAR-mediated membrane deformation. However, dimerization of endophilin also shows a regulatory activity to its SH3 (Src homology 3) domain, which locates at the C-terminal proximity to its BAR domain, because mutations disrupting dimerization impaired the binding of its partners [25]. Although MIM does not have an SH3 domain, it contains a PRD and a WH2 domain, which bind to cortactin and monomeric actin respectively. To analyse whether MIM dimerization has any effect on cortactin and actin binding, we analysed the association of MIM–Myc with cortactin and/or actin in the presence of MIM-S3 peptide, which has the most antagonistic activity among all of the peptides that we have tested. Although Myc antibody was able to detect the complex of MIM–Myc with cortactin and/or actin in the lysates of cells expressing MIM–Myc (Supplementary Figure S4 at http://www.BiochemJ.org/bj/446/bj460469add.htm), adding MIM-S3 peptide at 500 nM did not apparently interfere with the formation of the complex with either cortactin or actin. Thus MIM dimerization is dispensable for binding to cortactin and actin. The inability of MIM-S3 to interfere with cortactin and actin binding is probably due to the fact that the PRD and WH2 motifs locate remotely to the I-BAR domain by approximately 360 and 480 amino acids respectively (Figure 1A). In contrast, the SH3

Meng Cao, Tailan Zhan and Xi Zhan conceived the study, designed the experiments and wrote the paper. Min Ji and Xi Zhan are the principal investigators of the project.

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SUPPLEMENTARY ONLINE DATA

Dimerization is necessary for MIM-mediated membrane deformation and endocytosis

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Figure S1  GST–MIM-S1 interacts with recombinant MIM protein

(A) Coomassie Blue staining of purified GST–MIM-S1 and His6–MIM. Both forms of MIM proteins, in particular His6–MIM, display certain degrees of degradation. (B) GST–MIM-S1 at the concentrations indicated was incubated with 90 nM His6–MIM at 4°C for 2 h. After the interaction, the samples were subjected to pull-down using Ni-IDA (Ni2+–iminodiacetic acid) beads. The precipitates were separated by SDS/PAGE, and co-precipitated GST–MIM-S1 was detected by Western blotting using an anti-GST antibody.

Figure S2  Short synthetic peptides failed to inhibit MIM dimerization

MIM-S4, MIM-S5 and MIM-S6 peptides were analysed for the ability to inhibit dimerization of MIM–GFP and MIM–Myc as described in the Experimental section in the main text.

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Table S1  Inhibitory activity of synthetic peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Length (aa)</th>
<th>Region</th>
<th>Sequence</th>
<th>Inhibition of MIM dimerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIM-S3</td>
<td>33</td>
<td>I-BAR aa 57–89</td>
<td>AFQKVADMATNTRGGTREIGSALTRMCMRHSI</td>
<td>++++</td>
</tr>
<tr>
<td>MIM-S4</td>
<td>20</td>
<td>I-BAR aa 60–79</td>
<td>KVADMATNTRGGTREIGSAL</td>
<td>+</td>
</tr>
<tr>
<td>MIM-S5</td>
<td>12</td>
<td>I-BAR aa 64–75</td>
<td>MATNTRGGTREI</td>
<td>−</td>
</tr>
<tr>
<td>MIM-S6</td>
<td>6</td>
<td>I-BAR aa 67–72</td>
<td>NTRGGT</td>
<td>−</td>
</tr>
</tbody>
</table>

Figure S3  MIM-S3 inhibits dimerization of recombinant MIM proteins

(A) Coomassie Blue staining of purified His6–MIM and GST–MIM-I-BAR proteins. (B) His6–MIM (25 nM) was used to dimerize GST–MIM-I-BAR at various concentrations as indicated. The reactions were precipitated with glutathione beads, and the presence of His6–MIM in the pellets was detected by Western blotting using an anti-MIM antibody. (C) Dimerization of His6–MIM (25 nM) and GST–MIM-I-BAR (20 nM) was analysed in the presence of MIM-S3 at different concentrations. The K_d and IC_{50} values were calculated by Prism software.

Figure S4  MIM-S3 does not affect the association of MIM with cortactin and actin

HEK-293T cells were transiently transfected with and without MIM–Myc. The lysates of the transfected cells were subjected to immunoprecipitation with an anti-Myc antibody in the presence or absence of MIM-S3 (500 nM). The presence of cortactin and actin in the immunopellets was detected by Western blotting using anti-cortactin and anti-β-actin antibodies respectively.

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