Structural basis of filopodia formation induced by the IRSp53/MIM homology domain of human IRSp53

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The scaffolding protein insulin receptor tyrosine kinase substrate p53 (IRSp53), a ubiquitous regulator of the actin cytoskeleton, mediates filopodia formation under the control of Rho-family GTPases. IRSp53 comprises a central SH3 domain, which binds to proline-rich regions of a wide range of actin regulators, and a conserved N-terminal IRSp53/MIM homology domain (IMD) that harbours F-actin-bundling activity. Here, we present the crystal structure of this novel actin-bundling domain revealing a coiled-coil domain that self-associates into a 180 Å-long zeppelin-shaped dimer. Sedimentation velocity experiments confirm the presence of a single molecular species of twice the molecular weight of the monomer in solution. Mutagenesis of conserved basic residues at the extreme ends of the dimer abrogated actin bundling *in vitro* and filopodia formation *in vivo*, demonstrating that IMD-mediated actin bundling is required for IRSp53-induced filopodia formation. This study promotes an expanded view of IRSp53 as an actin regulator that integrates scaffolding and effector functions.

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Introduction

Cell shape changes and cell locomotion are essential in a great number of biological processes that occur in response to extracellular stimuli. The driving force for these processes is the dynamic reorganisation of the actin cytoskeleton. Actin filaments can form a diverse array of structures at the leading edge of migrating cells including lamellipodia, which are broad, sheet-like projections containing a network

of short branched filaments, and filopodia, which are thin needle-like projections containing bundles of long, parallel filaments (Small *et al*, 2002; Pollard and Borisy, 2003). Recent years have seen a great increase in our understanding of the proteins that control the formation of these actin structures in response to receptor-mediated stimuli. These include the filament nucleating Arp2/3 complex and its activators, the WASP/Scar/WAVE protein family as well as the Mena/VASP proteins, which promote filament elongation (Pollard and Borisy, 2003; Millard *et al*, 2004). The Rho family of GTPases also represent important actin regulators, notably Cdc42 and Rac, which promote the formation of filopodia and lamellipodia, respectively (Hall, 1998).

An actin regulator that has attracted a great deal of attention recently is the ‘insulin receptor tyrosine kinase substrate p53’ (IRSp53), also known as ‘brain-specific angiogenesis inhibitor 1 associated protein 2’ (BAIAP2) (Yeh *et al*, 1996). This ubiquitously expressed multidomain scaffolding protein has been implicated by several studies in filopodium formation (Govind *et al*, 2001; Krugmann *et al*, 2001; Yamagishi *et al*, 2004). At least one study has found evidence for a link between IRSp53 and lamellipodium formation (Miki *et al*, 2000). This evidence is further supported by the observation that IRSp53 localises to the tips of both filopodia and lamellipodia (Nakagawa *et al*, 2003). IRSp53 comprises a central CRIB motif followed by an SH3 domain, and WW and PDZ domain-binding motifs at the C-terminus (Figure 1A) (Govind *et al*, 2001; Krugmann *et al*, 2001; Hori *et al*, 2003; Soltau *et al*, 2004). Binding of activated Cdc42 to the CRIB motif enhances the ability of IRSp53 to promote filopodium formation, suggesting that IRSp53 may play a key role in the induction of filopodia by Cdc42 (Govind *et al*, 2001; Krugmann *et al*, 2001; Yamagishi *et al*, 2004). Interestingly, activation of IRSp53 by Rac1 does not depend on the presence of the CRIB motif, as this GTPase appears to bind to a distinct site located within the N-terminal 230 amino acids of IRSp53 (Miki *et al*, 2000; Miki and Takenawa, 2002). IRSp53 has been shown to bind, through its SH3 domain, to proline-rich regions of a number of known actin regulators, such as Scar2/WAVE2 (Miki *et al*, 2000; Miki and Takenawa, 2002), Mena (Krugmann *et al*, 2001), mDia1 (Fujiwara *et al*, 2000), ProSAP/shank (Bockmann *et al*, 2002; Soltau *et al*, 2002), espin (Sekerova *et al*, 2003) and eps8 (Funato *et al*, 2004). At least in a subset of cases, there is evidence to suggest that these interactions are dependent on IRSp53 being activated by binding active Cdc42 (Fujiwara *et al*, 2000; Krugmann *et al*, 2001; Soltau *et al*, 2002) or Rac (Miki *et al*, 2000; Miki and Takenawa, 2002). Furthermore, IRSp53 emerged as a binding partner of PDZ-domain-containing proteins such as PSD-95 in the post-synaptic density and MALs at cell–cell contacts (Hori *et al*, 2003; Soltau *et al*, 2004). This indicates that IRSp53 may

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Figure 1 Domain structure of IRSp53 and sequence alignment of IMD and BAR domains. **(A)** Schematic representation of the domain organisation of IRSp53. Vertical bars in grey are spaced by 100 residues. Domains are indicated by boxes in grey, and interaction motifs by boxes in white. **(B)** Sequence alignment of IMD and BAR domains using ClustalW at EBI (<http://www.ebi.ac.uk/clustalw>). Sequences and data base entries are as follows: IRSp53 NP_059344; IRTKS NP_061330; FLJ22582 NP_079321; MIM-B NP_055566; ABBA-1 NP_61239; GRAF NP_055886; centaurin-β2 NP_036419; arfaptin2 AAH00392; amphiphysin NP_001626. Symbols above the sequence alignment refer to IRSp53, indicating secondary structure assignment, involvement in dimer contact surface (=) and residue numbers. Asterisks in magenta indicate the mutation sites. Below the sequence alignment, secondary structure of the BAR domain of amphiphysin, and residues contributing to the dimer contact surface area (hyphen in magenta) of the BAR domain are indicated. Sequence conservation is highlighted by colouring (ClustalX), magenta (Glu, Asp), cyan (Trp, Met, Phe, Leu, Ile, Val, Ala), dark cyan (His, Tyr), green (Gln, Asn, Ser, Thr), red (Lys, Arg), yellow (Pro) and brown (Gly).

play a central role in a wide range of actin regulating complexes.

It has recently been established that the N-terminal 250 amino acids of IRSp53 form a conserved domain that is able to bundle actin filaments (Yamagishi *et al*, 2004).

The IRSp53 and MIM homology domain (IMD) occurs in IRSp53 and the B isoform of 'missing in metastasis' (MIM-B), a recently described actin regulator, as well as in at least three uncharacterised mammalian proteins (Figure 1B), and orthologues exist in both *Drosophila* and *Caenorhabditis*

Table I Data collection, phasing and refinement

Data collection	Inflection point	Peak	High-E remote
Space group (molecules/a.s.u.)		$P2_1$ (2)	
a (Å)		49.9	
b (Å)		64.2	
c (Å)		74.6	
β (deg)		106.2	
Station ^a		14.2	
Wavelength (Å)	0.9781	0.9776	0.9649
f' , f''	−10.3, 3.5	−7.2, 6.1	−1.8, 3.5
Resolution range (Å)	47.7–2.2	47.8–2.2	47.8–2.2
Last shell (Å)	(2.32–2.20)	(2.32–2.20)	(2.32–2.20)
R_{merge}^b : overall (last shell)	0.051 (0.219)	0.056 (0.192)	0.043 (0.225)
Observations: overall (last shell)	168716 (23196)	169779 (23035)	85026 (12164)
Unique reflections: overall (last shell)	22395 (3177)	22459 (3133)	22530 (3259)
Mean (I)/s.d. (I): overall (last shell)	27.5 (7.9)	25.9 (8.4)	21.2 (5.8)
Completeness: overall (last shell)	97.7 (95.2)	97.4 (93.7)	98.0 (97.3)
Multiplicity: overall (last shell)	7.5 (7.3)	7.6 (7.4)	3.8 (3.7)
Anomalous completeness	97.8 (95.2)	97.4 (93.6)	96.8 (93.0)
Anomalous multiplicity	3.9 (3.7)	3.9 (3.7)	1.9 (1.8)
<i>Phasing</i>			
Number of selenium atom sites		15	
Resolution range (Å)		30.0–2.2	
Figure of merit		0.64	
<i>Structure refinement</i>			
Resolution range (last shell) (Å)	47.8–2.2 (2.32–2.20)		
R -factor (last shell) (%)	22.8 (24.9)		
R_{free} (last shell) (%) ^c	26.7 (33.0)		
<i>Total number of</i>			
Nonhydrogen atoms	4164		
Protein atoms	3943		
Water molecules	221		
<i>r.m.s.d.^d</i>			
Bond length (Å)	0.010		
Bond angle (deg)	1.1		
Main chain B -factors (Å ²)	0.8		
Side chain B -factors (Å ²)	2.0		
Wilson B -factor (Å ²)	35.3		
Average B -factor protein atoms (Å ²)	36.2		
Average B -factor solvent atoms (Å ²)	36.5		
<i>Ramachandran statistics^e</i>			
Most favoured regions (%)	96.5		
Additionally allowed regions (%)	3.1		
Generously allowed regions (%)	0.4		
Disallowed regions (%)	0.0		

^aBeamline designation refers to the SRS Daresbury.

^b $R_{\text{merge}} = \sum (|I| - \langle I \rangle) / \sum |I|$.

^c R_{free} calculated using 5% of total reflections omitted from refinement.

^dr.m.s.d. = root mean square deviations from ideal bond lengths/angles with respect to (Engh and Huber, 1991), and of B -factors between bonded atoms.

^eRamachandran statistics calculated using PROCHECK (CCP4, 1994).

elegans (Woodings *et al*, 2003; Yamagishi *et al*, 2004). The IMD constitutes the only domain shared between IRSp53 and MIM-B, and the IMD of either protein, in isolation, is able to bundle actin fibres *in vitro* and to induce filopodia formation *in vivo* (Yamagishi *et al*, 2004).

We report here the crystal structure of the IMD of IRSp53 determined to 2.2 Å resolution. This structure together with site-directed mutagenesis and functional assays provides insights into the mechanism of action of IRSp53 and that of other IMD-containing proteins.

Results

Overall structure and topology

The crystal structure of the IMD of human IRSp53 (residues 1–250) has been determined *de novo* by multiwavelength anomalous dispersion and refined to a resolution of 2.2 Å (Table I). The experimental density map (Figure 2A) delineates the entire polypeptide with the exception of two N-terminal and two C-terminal residues, the former arising from the expression construct. The asymmetric unit of the monoclinic unit cell comprises two IMD monomers

Figure 2 Structure of the IMD of human IRSp53. (A) Stereo diagram of the electron density derived from the three-wavelength MAD experiment contoured at 0.8σ and superimposed with the refined model. Shown is the core segment of the IMD signature sequence ($^{189}\text{EERRR}^{193}$). Bonds and atoms are coloured according to atom type (yellow/grey: carbon; red: oxygen; blue: nitrogen). Selected residues are indicated in single letter code. Chains A and B are distinguished by the colour of the carbon bonds. (B) Two orthogonal views, parallel and perpendicular to the noncrystallographic two-fold axis, of the IMD dimer in ribbon and molecular surface representation. The ribbon diagrams are colour ramped blue to green (chain A) and yellow to red (chain B). The mutation sites are indicated for one of the monomers in single letter code, and the location of the IMD signature sequence is highlighted with side chains in grey. Vertical dashed lines indicate the core region of the dimer. N- and C-termini of chains A and B are indicated in italics. (C) Packing of the IMD dimer in the unit cell. (D) Structural superposition of the IMD dimer with the BAR domain dimer (grey) of amphiphysin (pdb: 1uru, (Peter *et al*, 2004)). Figures 2 and 3 were generated using RIBBONS (Carson, 1997), Swiss-PDB Viewer (Guex and Peitsch, 1997), O (Jones *et al*, 1991) and GRASP (Nicholls *et al*, 1991).

(Figure 2C) forming a zeppelin-shaped dimer with a very extensive dimer interface (Figures 2B and 3A). The dimer is about $25\text{ \AA}$ in diameter and measures $180\text{ \AA}$ in length, with the dyad relating the two monomers roughly parallel to the crystallographic *b*-axis. The IMD monomer folds as a coiled coil of three extended α -helices and a shorter C-terminal helix that is separated by an 8-residue linker from the preceding helix 3. Helix 4 packs tightly against the other three helices, and thus represents an integral part of the domain. The fold of the IMD monomer closely resembles that of the BAR (Bin/amphiphysin/Rvs) domain of arfaptin2 and amphiphysin

Figure 3 The dimer interface and properties of the molecular surface of the IMD dimer. (A, B) Molecular contact surface (yellow) of the IMD (A) and amphiphysin BAR domain (B) dimers, calculated using SwissPDB-Viewer (Guex and Peitsch, 1997). (C) Molecular surface of the IMD dimer oriented with the dyad parallel to the viewing direction. The surface area is coloured according to sequence conservation among the five mammalian IMD sequences in Figure 1. The colour shading is based on the Q-score calculated by ClustalX (Thompson *et al*, 1997), whereby deep blue indicates identity ($Q=100$). The top and bottom panels show the N- and C-terminal faces of the dimer, respectively. The C in italics on the lower panel indicates the location of the C-termini on the IMD dimer. (D) Molecular surface of the IMD dimer oriented as in panel (C) and coloured according to electrostatic surface potential, colour ramped from $-15\text{ k}_B T/e$ (red) to $+15\text{ k}_B T/e$ (blue).

sin (Tarricone *et al*, 2001; Peter *et al*, 2004), but the dimers show markedly different shapes (Figure 2D). The C-terminus of helix 4 is adjacent to the N-terminus of helix 1 of the opposite monomer, with the domain termini falling onto the boundary of the central region, an area approximately defined by the extent of the dimer interface. Helices 2 and 3 protrude beyond the central region, which is made up of a 6-helix bundle, and the poorly ordered 2–3 loops demarcate the extreme ends of the dimer.

The dimer interface

The dimer interface is very extensive burying about $5800\text{ \AA}^2$ of solvent-accessible surface, with a contact surface area of

Figure 4 *In vitro* characterisation of the IMD of IRSp53 and interaction with F-actin. (A) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of purified IMDwt (wt) and IMD_{C230A} (C230A) analysed under reducing (+ DTT) and nonreducing (–DTT) conditions after heat denaturation. All gels in this figure were stained with Coomassie blue and positions of molecular weight markers are indicated in units of 10^3 Da. (B) Sedimentation coefficient distributions of IMDwt and IMDmut. The main peak for both is at 3.25s, while some higher order aggregate is observed for the mutant at 4.5s. (C) Circular dichroism spectroscopy of wild-type (thick line) and mutant (thin line) forms of the IMD. (D) High-speed cosedimentation assay of the interaction of wild-type (wt) and mutant IMD (mut) with F-actin (2.5 μ M). Pellet fractions were analysed by SDS–PAGE and the band intensity of the IMD was measured densitometrically and normalised to actin. (E) Low-speed co-sedimentation assay of F-actin bundling. Pellet (P) and supernatant (S) fractions were analysed by SDS–PAGE. (F) Fluorescence microscopy-based F-actin-bundling assay. Cy3-labelled F-actin (1 μ M) was incubated with 5 μ M wt or mut IMD and imaged using a fluorescence microscope (scale bar = 20 μ m).

$\sim 1350 \text{ \AA}^2$ per monomer (Figure 3A). By comparison, the crescent-shaped dimer of the BAR domain of amphiphysin buries about $3500 \text{ \AA}^2$ of solvent-accessible surface, with $\sim 620 \text{ \AA}^2$ contact surface area per monomer (Figure 3B). Hydrophobic and polar surfaces contribute to nearly equal parts to the contact surface area, which involves all four helices (Figure 1B). The interface encloses a substantial cavity of $\sim 930 \text{ \AA}^3$ that contains several water molecules. Cysteines 195 and 230 of opposite chains are juxtaposed such that disulphide bond formation across the dimer interface is geometrically possible, but suppressed due to the presence of 5 mM dithiothreitol (DTT) in the acidic crystallisation buffer (pH 4.6). Disulphide bond formation is indeed observed on a denaturing SDS–acrylamide gel under

nonreducing conditions, but not when Cys230 is mutated to alanine (Figure 4A). This provides independent evidence that the dimer interface revealed by the crystal structure is likely physiologically relevant. In order to verify the oligomerisation state of the IMD in solution, we performed sedimentation velocity experiments in the presence of DTT (mirroring the crystallisation conditions), which indicated a single molecular species at a sedimentation coefficient of 3.25s (Figure 4B). Fitting the data to a single species model yields a molecular weight of 56 792 Da (95% confidence interval: 51 517–60 642), representing twice the calculated molecular weight of the IMD monomer, 28 563 Da. No other species can be detected.

In agreement with previous data (Yamagishi *et al*, 2004), we also observed self-association of full-length IRSp53 in COS7 cell lysates as myc-tagged IRSp53 co-immunoprecipitated with HA-tagged IRSp53 (data not shown). Thus, the IMD forms a dimer in solution and is likely responsible for the observed dimerisation of the full-length protein *in vivo*.

Structural differences between monomers

The present model of the IMD dimer has been constructed without applying noncrystallographic symmetry restraints throughout phasing, model building and refinement, allowing to detect potential conformational differences between the monomers. The two subunits superimpose very well within the core region (residues 1–130 and 173–248, root mean square deviation (r.m.s.d.) $0.57 \text{ \AA}$ for 206 C α positions) that is defined by the extent of the dimer interface. In contrast, when all 248 C α positions of the present model are included to calculate the superimposition, the r.m.s.d. increases almost three-fold to $1.5 \text{ \AA}$, reflecting a significant level of conformational freedom for those parts of helices 2 and 3 that extend beyond the core region. Accordingly, drastic differences between the monomers in terms of side chain or backbone conformation fall outside the core region, with one notable exception: the side chain of Tyr115, which is located adjacent to a kink in helix 2. In chain A, the Tyr115 hydroxyl is observed to form a tight hydrogen bond with Glu189 in helix 3. In chain B, this side chain is rotated by almost 180° away from Glu189 and interacts with a symmetry-related molecule. The side chain rotation opens a sizeable crevice in the surface of the IMD that exposes the guanido group of Arg193 to solvent. We note that both Glu189 and Arg193, albeit not Tyr115, are invariant residues belonging to the IMD signature motif (see below). This fact may suggest that the observed conformational difference might be of functional significance, a question that is not further addressed in this study.

Sequence conservation

The surface area of the IMD dimer displays a marked polarity in terms of sequence conservation with respect to the five IMD sequences in Figure 1B. When the dyad axis of the dimer is oriented perpendicular to the plane of the paper, the N- and C-termini of the IMD dimer are displayed on the front and back faces of the dimer, respectively (Figure 3C, upper and lower panel). The N-terminal face shows extended patches of invariant solvent-exposed residues, while the C-terminal face is almost completely devoid of invariant or highly conserved residues. The two extended, dyad-related patches of invariant residues seen on the N-terminal side include Met21, Pro26,

Glu126, Lys136, Lys143 and Lys147, all of which are part of conserved segments in the sequence of the IMD (Figure 1B). These patches surround two distinct voids in the molecular surface of the IMD dimer, which are bounded by the N-terminus on the core side of the dimer, and by the cluster of conserved basic residues (Lys142–Lys147) on the other side. Interestingly, the signature sequence of the IMD (residues 186–197, ALXEE[RK][RG]RFCX(0,1)F[IL]; Yamagishi *et al*, 2004) is mostly buried in the dimer. Residues in its core segment ¹⁸⁹EERRR¹⁹³ make contacts with other conserved parts of the opposite monomer (Figure 2A and B), notably with the loop linking helices 1 and 2, and the 8-residue linker separating helices 3 and 4. For instance, Glu190 forms strong hydrogen bonds with the backbone amides of Gln67 and Gly68 in the 1–2 loop (Figure 2A); Arg191 forms salt bridges to residues Glu71 and the carbonyl oxygen of Asp232; Arg193 makes van der Waals contacts with Phe24 and Ile20 of the same monomer, ‘pushing’ into a kink of helix 1.

The polarity in terms of sequence conservation is mirrored, albeit to a lesser degree, by a noticeable polarity of the surface charge distribution. The extreme ends of the dimer present a strong positive surface potential on the N-terminal side, but do so to a much lesser degree on the C-terminal side. Elsewhere on the surface, the charge distribution appears more or less even, with positive charges slightly prevailing overall (Figure 3D).

Identification of residues involved in actin binding

At the extreme ends of the dimer are a cluster of basic surface residues that are well conserved between the five known mammalian IMDs, suggesting that these were likely to be significant to IMD function. Electron microscopy of IMD-induced F-actin bundles reveals that the centres of adjacent F-actin fibres are spaced about 11 nm apart (Yamagishi *et al*, 2004) and this distance correlates with the distance between the conserved basic patches. Moreover, the surface charge of F-actin fibres is highly acidic, suggesting that basic surface areas of the IMD dimer are likely to be involved in F-actin binding. We therefore targeted the extreme ends of the dimer in a mutational analysis. Mutant constructs were first made based on plasmids encoding full-length IRSp53 and assayed *in vivo*. Mutations that showed compromised filopodia formation were then followed up by making the same mutations in plasmid constructs encoding the IMD alone. We found initially that mutant forms of IRSp53, where only a single site was altered in the Lys142–Lys147 cluster, were indistinguishable from wild-type IRSp53 in the cell-based assay (data not shown). In contrast, altering all four lysines (142, 143, 146 and 147) to glutamic acid dramatically affected the behaviour of both full-length IRSp53 and the IMD.

Wild-type (IMDwt) and mutant (IMDmut, K142E, K143E, K146E, K147E) forms of the IMD of IRSp53 were expressed as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* and purified to homogeneity in their tag-free form. IMDwt and IMDmut were indistinguishable in circular dichroism spectroscopy experiments (Figure 4C). While the sedimentation velocity experiments indicated a slight tendency of the mutant to aggregate (peak at 4.5s, Figure 4B), the predominant species in solution is still the dimer peak and, crucially, its sedimentation coefficient is unchanged from that of wild-type IMD, indicating clearly

that the global conformation of the dimer is unchanged by the site-directed mutagenesis. Thus, the mutation did not affect the association state or the fold of the IMD. F-actin binding by IMDwt and IMDmut was analysed using a filament cosedimentation assay varying the concentration of IMD. The pellet fractions were analysed by SDS–polyacrylamide gel electrophoresis and the gels evaluated densitometrically (Figure 4D). The equilibrium dissociation constant K_d derived from these binding curves is 5 and 10 μ M for IMDwt and IMDmut, respectively. While the nature of this assay limits the accuracy of the K_d values obtained, it unambiguously demonstrates that the weakened binding affinity of IMDmut for F-actin.

Actin bundling was assayed *in vitro* using a low-speed cosedimentation assay. In the absence of IMD, 25 % of F-actin was typically found in the pellet. Addition of IMDwt resulted in a concentration-dependent increase of the proportion of F-actin in the pellet, demonstrating filament bundling (Figure 4E, left panels), whereas addition of IMDmut had no effect on the partitioning of F-actin between pellet and supernatant (Figure 4E, right panels).

In an independent assay, F-actin coupled to a fluorescent dye (Machesky and Hall, 1997) was incubated with IMDwt and IMDmut at various concentrations and then visualised by fluorescence light microscopy. The presence of IMDwt resulted in the formation of visible actin bundles (Figure 4F). Single bundles as well as branched networks of bundles were observed. The number and size of bundles increased with IMDwt concentration as did the extent of branching. No bundles were observed at any concentration of IMDmut tested. These data demonstrate that although IMDmut is correctly folded and present as a dimer (Figure 4B and C), it cannot bundle actin fibres due to the disruption of the actin-binding sites.

Mutation of basic patches of IMD abrogates filopodium formation by IRSp53

Several groups have observed that overexpression of IRSp53 in a range of cell lines results in the formation of filopodia. Overexpressing myc-tagged IRSp53 in COS7 cells, we observed long filopodia that were often branched (Figure 5A). While filopodia were observed in untransfected COS7 cells, these were usually short (<10 μ m) and clearly distinguishable from those induced by IRSp53 overexpression, which were typically 10–60 μ m in length. Myc-tagged IRSp53 was localised throughout the filopodia and often concentrated at the tip. As described previously (Yamagishi *et al*, 2004), we observed that expression of the isolated IMD of IRSp53 also resulted in the formation of filopodia (Figure 5B), while deletion constructs of IRSp53 that lacked the IMD were unable to induce filopodia formation (data not shown). IMD-induced filopodia tended to be less frequently branched than those induced by full-length IRSp53. In marked contrast to the wild-type constructs, myc-tagged forms of IMDmut or of full length IRSp53 carrying the analogous mutation failed to display filopodia formation above the level of untransfected cells (Figure 5). This clearly demonstrates that IRSp53 mutants whose actin-binding and actin-bundling activity is compromised cannot induce filopodia, establishing a molecular basis of IRSp53-induced filopodia formation.

Figure 5 Analysis of IRSp53 and IMD overexpression in COS7 cells. (A) Plasmid constructs encoding myc-tagged forms of either IRSp53 (wild-type) or IRSp53 (mutant) were transfected into COS7 cells for 24 h, fixed with paraformaldehyde and stained with TRITC-phalloidin and anti-myc, followed by a fluorescent secondary antibody. Coverslips were visualised by fluorescence microscopy. (B) As (A), but cells were transfected with plasmid constructs encoding myc-tagged forms of IMDwt or IMDmut (scale bars in (A) and (B) = 20 μ m). (C) Cell counts comparing filopodia formation in transfected versus untransfected COS7 cells. Error bars represent standard deviations from three independent blind experiments counting approximately 35 transfected and untransfected cells for each condition. Cells were scored positive for filopodia if they showed at least five filopodia with a length greater than 10% of the cell diameter, corresponding to approximately 10 μ m.

Discussion

The IMD constitutes a novel actin-bundling domain that is essential for IRSp53 function. Our results confirm previously published data that the IMD is required for IRSp53-induced

filopodia formation, and that this domain crosslinks F-actin to form bundles of filaments. We show here, for the first time, that filopodia formation induced by full-length IRSp53 or the IMD in isolation is compromised when the actin-binding sites in the IMD are mutated. This indicates that IMD-mediated actin-binding and actin-bundling activity is required for IRSp53-induced filopodium formation. Chemical crosslinking and co-immunoprecipitation data have previously suggested IMD-mediated dimerisation of IRSp53 (Yamagishi *et al*, 2004). Analytical ultracentrifugation in sedimentation velocity mode confirms that the IMD exists as a dimer in solution, and co-immunoprecipitation data indicate dimerisation of full-length IRSp53 *in vivo*. The crystal structure reveals that IMD dimerisation is mediated by a vast interface, of which about 50% are contributed by hydrophobic residues, strongly suggesting that this interface is physiologically relevant. Together, these data argue that F-actin-bundling activity is mediated through the bivalent nature of the IMD dimer. The mutational analysis maps the actin-binding sites to the extreme ends of the dimer. The binding sites and the C-termini of the IMD subunits fall on opposite sides of the dimer, reconciling space requirements of the actin filaments and the C-terminal domains of IRSp53. The orientation in which the IMD dimer binds F-actin is not known. In one possible scenario, the two-fold axis of the dimer is oriented perpendicular to the filament axes, that is, in the orientation of Figure 3C, the filament axes would run vertically along the plane of the paper on the N-terminal side of the dimer. In this binding mode, the IMD would present surface patches with opposite polarity to parallel F-actin filaments. The question of surface polarity can be resolved, if it is assumed that the two-fold axis of the dimer is oriented parallel to the fibre axes. With respect to Figure 3C, the filaments would run perpendicular to the plane of the paper, one above and one below of the IMD dimer. In this scenario, the extreme ends of the dimer could contact the underside of one of the outer domains of actin.

The IMD differs significantly in structure from that of other 'dedicated' actin-binding or actin-bundling domains. Coiled-coil domains frequently mediate protein oligomerisation both in actin-crosslinking proteins and elsewhere, for instance, in the rod domain of α -actinin (Ylanne *et al*, 2001), or the C-terminal domain of cortexillin (Faix *et al*, 1996; Burkhard *et al*, 2000). F-actin binding has been reported for a subset of the coiled-coil spectrin-like repeats of the rod domains of dystrophin and utrophin (Amann *et al*, 1998; Rybakova *et al*, 2002). However, both proteins also contain a 'dedicated' actin-binding domain based on the tandem repeat of the calponin homology domain (Carugo *et al*, 1997). This tandem repeat is conserved among actin-binding domains of the utrophin superfamily, and the F-actin-binding sites in the rod domain have been suggested to help increase overall binding affinity. The actin-bundling proteins fimbrin and fascin are of particular interest, as both have been implicated in filopodia formation (Small *et al*, 2002). The actin-bundling core of fimbrin (Klein *et al*, 2004) is composed of four calponin homology domains, a duplication of the calponin homology domain tandem repeat. In contrast, fascin (pdb entry 1dfc, Fedorov *et al*, unpublished) is composed of four repeats of the β -trefoil domain (Ponting and Russell, 2000). Thus, the elementary structural unit in the actin-binding domains of both fimbrin and fascin is a compact globular

domain. Based on a DALI search (<http://www.ebi.ac.uk/dali>; Holm and Sander, 1998), the closest structural relative of the IMD representing a 'dedicated' F-actin-binding domain appears to be the tail piece of vinculin (Bakolitsa *et al*, 1999, 2004; Izard *et al*, 2004). Ranked 17 in the DALI search with a Z-score of 7.4 (compared to 12.8–11.8 for the top hits, the amphiphysin and arfaptin2 BAR domains), the vinculin tail piece forms a compact 5-helix bundle that, upon activation by talin, binds F-actin (Bakolitsa *et al*, 2004; Izard *et al*, 2004). However, vinculin has no actin-bundling activity.

The similarity of the IMD to the BAR domain, a functional module serving both as a sensor and inducer of membrane curvature (Peter *et al*, 2004), is intriguing. Despite displaying different shapes and being functionally distinct, the two domains form superimposable dimers (r.m.s.d. 1.9 Å for 226 of 496 C α positions, Figure 2D) and present both prominent positive surface charges at the extreme ends. Hallmarks that distinguish the IMD from the BAR domain include the fourth helix at the C-terminus of and the IMD signature motif. Helix 4, an integral part of the IMD, is chiefly responsible for the much larger dimer interface of the IMD and also appears to stabilise the extended, straight conformation of helices 2 and 3. The core segment of the IMD signature motif (¹⁸⁹EERRR¹⁹³), which is not conserved in BAR domains, is engaged in a number of intradimer interactions, notably with the 8-residue linker between helices 3 and 4, a structural element not present in the BAR domain.

While binding of the IRSp53-SH3 domain to the proline-rich regions of various actin cytoskeleton regulating proteins depends on activation by Cdc42 or Rac1 (Miki *et al*, 2000; Krugmann *et al*, 2001; Bockmann *et al*, 2002; Miki and Takenawa, 2002; Soltan *et al*, 2002), it is not known whether these small G proteins directly affect IMD-mediated actin-bundling activity. Based on the present structure, it is conceivable that Cdc42 binding to IRSp53 may involve (nonspecific) contacts with the IMD, as the Cdc42-binding site, the CRIB motif (residues 266–279), is located only 16 residues C-terminal to the IMD. Rac1, in contrast, has been reported to bind directly to the IMD (Miki *et al*, 2000; Miki and Takenawa, 2002), consistent with our own experimental data (not shown). A model for Rac1 binding to the IMD can be readily derived by superimposing the IMD structure with the Rac1-arfaptin2 complex (Tarricone *et al*, 2001). Sequence conservation at the putative Rac1-binding site is weak, but the model entails only minor steric clashes, placing Rac1 centrally on the N-terminal side of the IMD dimer. While Rac1 has been shown to enhance IRSp53-induced filopodia formation *in vivo* (Yamagishi *et al*, 2004), we have not found that constitutively active Rac1 significantly enhances IMD-mediated bundling activity *in vitro* (data not shown). However, the assay employed, a low-speed cosedimentation assay, might not be sensitive enough to detect subtle regulatory effects.

It has previously been suggested that IRSp53 functions as a scaffolding protein, recruiting other signaling proteins and effectors to sites of filopodia assembly. If there is indeed, as our data suggest, a direct mechanistic link between IMD-mediated actin bundling and IRSp53-induced filopodia formation, IRSp53 would have to be considered a protein that integrates regulatory and effector functions. Mena/VASP proteins, which have been implicated in filopodium formation and associate with IRSp53 through the IRSp53-SH3 domain,

promote filament lengthening by inhibiting barbed end capping (Bear *et al*, 2002; Lebrand *et al*, 2004; Mejillano *et al*, 2004). It is conceivable that filopodium formation in response to activation by Rho-family GTPases is facilitated by the combined effect of Mena-controlled inhibition of barbed end capping and IRSp53-induced F-actin bundling. Pre-existing bundles in the filopodium may and then be further stabilised by other actin-bundling proteins such as fimbrin and fascin. The presence of a range of protein interaction motifs within IRSp53 and the large set of identified IRSp53 binding partners further underscore the proposed dual function of IRSp53 as a scaffolding and effector protein. They also suggest that the regulatory role of IRSp53 may extend beyond the initiation of filopodia formation.

Materials and methods

DNA constructs

Human IRSp53 in the mammalian expression vector pRK5myc was obtained from Dr Alan Hall (LMCB, London) (Krugmann *et al*, 2001). This was used as a template to amplify the region encoding the IMD (amino acids 1–250) of IRSp53 by polymerase chain reaction. Sites for restriction enzymes *Bam*HI and *Eco*RI were introduced into the forward and reverse primers, respectively, and these sites were used to clone the construct into pRK5myc and pGEX4T2 (Amersham Biosciences) plasmids. Site-directed mutagenesis was performed using the QuikChange procedure (Stratagene) following the manufacturer's recommendations. All primers were purchased from Sigma-Genosys. DNA sequencing of all constructs was performed by the University of Birmingham Functional Genomics laboratory.

Protein expression and purification

Plasmids pGEX4T2 containing the coding sequence of the IMD were transformed into the methionine-auxotroph strain *E. coli* B834(DE3), obtained from Professor JB Jackson (University of Birmingham), for the purpose of crystallographic structure determination, or into *E. coli* BL21(DE3) for the biochemical assays. The transformants were used to inoculate 4 l of minimal M9 medium (Sambrook *et al*, 1998) supplemented with 50 µg/ml selenomethionine (Fisher Scientific) and 100 µg/ml ampicillin (Sigma), while unlabelled protein was produced in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin. Cultures were grown at 37°C to OD₆₀₀ 0.45, and protein expression was induced with 1 mM IPTG (Melford labs) for 14 h at 25°C. Cells were lysed by sonication in PBS/1% Triton X-100 containing a protease inhibitor cocktail. The cleared sonicate was then passed three times over a glutathione-agarose column. The column was washed with the sonication buffer followed by thrombin buffer (150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 5 mM MgCl₂, 2.5 mM CaCl₂, 1 mM DTT). Thrombin (40 U) (Calbiochem) was added in one column volume of thrombin buffer and the column incubated for 15 h (4°C). Thrombin-cleaved IMD was eluted with thrombin buffer and dialysed into 140 mM NaCl, 20 mM Hepes (pH 7.0), 5 mM MgCl₂, 2.5 mM CaCl₂, 1 mM DTT, bound to a carboxymethyl-sepharose column and eluted with a linear gradient of 140–500 mM NaCl. Fractions were analysed by SDS-PAGE, and those containing pure IMD were pooled and concentrated to 50 mg/ml protein using Vivaspin 15 (Vivascience) and centricon 10 (Amicon) ultrafiltration columns.

Crystallisation and structure determination

Crystals of the selenomethionine-labelled form of the IMD of IRSp53 were grown by hanging drop vapour diffusion over a reservoir containing 15–18% (w/v) polyethylene glycol (PEG) 6000, 0.1 M sodium acetate (pH 4.6), 15–70 mM ammonium acetate, 5 mM DTT and small concentrations of either sucrose (2–6% (w/v)) or ethylene glycol (10–12% (v/v)). Crystals appeared and grew to final size over night, but showed signs of decay only a few days later. Therefore, crystals were flash frozen in liquid nitrogen promptly as follows. High-quality diffraction patterns were obtained by soaking crystals in a cryoprotection buffer based on the

reservoir solution, but with PEG 6000 increased to 30% (w/v) and 20% ethylene glycol or sucrose prior to data collection from flash frozen crystals. Crystals were in space group $P2_1$ (Table I) with two molecules in the crystallographic asymmetric unit. A three-wavelength MAD data set was recorded on station 14.2 at SRS Daresbury (Warrington, UK) integrated and scaled using MOSFLM/SCALA (Leslie, 1992; CCP4, 1994). An initial set of 10 out of 16 possible selenium positions was determined from the anomalous differences of the 'absorption peak' data using SnB v2.1 (Weeks and Miller, 1999). Five additional positions, including that of the initial Met residue of chain A, were located by SOLVE (Terwilliger and Berendzen, 1999) and included in the phase calculation (30–2.2 Å), followed by solvent flattening using RESOLVE (Terwilliger, 2002). The resulting map was of a very good quality and allowed to unequivocally build the entire backbone (chain tracing using ARP/WARP (Morris *et al*, 2002) combined with manual building in O (Jones *et al*, 1991)), except for two residues at the C-terminus of both chains A and B, and two N-terminal residues that arose from the expression construct. Side chains were added manually and the model was refined (CNS 1.1 (Brünger *et al*, 1998), REFMAC5 (Murshudov *et al*, 1997)) against the peak wavelength data restrained by experimental phases. Several rounds of manual rebuilding interspersed with refinement lowered the free *R*-factor (5% of reflections) to 29.5%, at which point water molecules were added. Met residues were modelled as seleno-methionine, whereby the free *R* values were only marginally different when using sulphur-methionine instead. This probably reflected the fact that at present anomalous scattering factors cannot be modelled in the CCP4 distribution of REFMAC5 (Murshudov *et al*, 1997). Density for loops 2–3 is weak and a number of side chains in this region could not be built with confidence, in particular in chain A. The final model comprises 496 protein residues (248 each in chains A and B) and 221 water molecules. Temperature factors were refined applying a TLS correction (Winn *et al*, 2001) (two groups) and the minimisation converged to a free *R* value of 26.7% with excellent stereochemistry (Table I).

Sedimentation velocity

Sedimentation velocity experiments were carried out in a Beckman XL-A analytical ultracentrifuge (Beckman Coulter, Palo Alto, CA, USA) equipped with absorbance optics. Protein samples were dialysed into storage buffer as above including 1 mM DTT and loaded into cells with two channel Epon centre pieces and quartz windows. Data were recorded at 40 000 r.p.m., 20°C, using an An50Ti rotor and an absorbance wavelength of 280 nm, with scans taken every 6 min. Solvent density was measured using an Anton Paar DMA 5000 high-precision density metre. Partial specific volumes were calculated using the program SEDNTERP (Laue *et al*, 1992). Data were analysed using the program SEDFIT (Schuck, 2000). Sedimentation coefficient distributions were calculated using the Lamm equation modelling implementing maximum entropy regularisation. A total of 95 scans for each sample were analysed, representing the full extent of sedimentation of the sample. For the wild-type sample, data were fitted to a single species model using finite element solutions to the Lamm equation (Schuck, 1998).

F-actin-binding and F-actin-bundling assays

G-actin was purified from rabbit muscle as described previously (Spudich and Watt, 1971). G-actin was maintained in G-buffer: 2 mM Tris-Cl (pH 8.0), 0.2 mM ATP, 0.5 mM DTT and 0.2 mM CaCl_2 . Polymerisation of the actin was achieved by addition of 50 mM KCl, 2 mM MgCl_2 and 0.1 mM EGTA, the resulting buffer being F-buffer. Concentrations measures given for F-actin in this paper refer to the initial concentration of G-actin prior to polymerisation. Plasmid constructs of wild-type and mutant forms of IMD were cloned into the pGEX4T2 expression vector and transformed into *E. coli*

BL21(DE3) cells and purified as described in the above section. Proteins were dialysed against F-buffer and centrifuged at 435 000 g for 60 min at 4°C, immediately prior to use in actin-binding/bundling experiments. Protein concentrations were measured using the Biorad protein assay and confirmed by SDS-PAGE analysis.

Actin-binding experiments. F-actin (2.5 μM) was incubated with a range of concentrations (0.5–10 μM) of either wild-type or mutant IMD in F-buffer for 30 min on ice, and then centrifuged at 350 000 g (30 min, 4°C). Supernatants were removed, pellets rinsed with F-buffer then resuspended in a volume of F-buffer equal to that of the supernatant. Samples were analysed by SDS-PAGE and Coomassie staining, and quantified by densitometry using GENETOOLS (Syngene), and normalised with respect to the F-actin band intensity.

Actin-bundling experiments. F-actin (5 μM) was incubated with varying concentrations of either wild-type or mutant IMD in F-buffer at room temperature for 1 h, and then centrifuged at 16 000 g for 30 min at room temperature. Supernatants were removed and pellets resuspended in a volume of F-buffer equal to that of the supernatant.

Fluorescence microscopy of actin bundling. Rabbit muscle G-actin was labelled using FluoroLink Cy3-reactive dye (Amersham Biosciences) as described previously (Machesky and Hall, 1997). This was mixed with unlabelled G-actin, such that 15% of monomers carried the Cy3 label. The actin was polymerised and varying concentrations of the labelled F-actin was mixed with varying concentrations of wild-type and mutant IMD in F-buffer and incubated at room temperature for 1 h. The samples were then mounted between a slide and coverslip and imaged by fluorescence microscopy using a Zeiss Axioskop2 and Hamamatsu C47442-95 camera. Images were processed using Openlab (Improvision) and Photoshop 7.0 (Adobe).

Cell culture

COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and antibiotics. Cells were seeded onto glass coverslips and transfected 24 h later using Gene Juice (Novagen) according to the manufacturer's recommendations. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilised in PBS containing 0.1% Triton X-100. Cells were stained with TRITC-phalloidin (Sigma) and anti-myc monoclonal antibody 9E10 (obtained from Cancer Research, UK), followed by an anti-mouse IgG secondary antibody conjugated to Alexa 488 (Molecular Probes).

Protein Data Bank (PDB) ID code

The coordinates of the IMD of IRSp53 have been deposited at the PDB (<http://www.rcsb.org/pdb>) under accession number 1Y2O.

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