Structural basis for regulation of Arp2/3 complex by GMF

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The Arp2/3 complex mediates formation of complex cellular structures such as lamellipodia by nucleating branched actin filaments. Arp2/3-complex activity is precisely controlled by over a dozen regulators, yet the structural mechanism by which regulators interact with the complex is unknown. GMF is a recently discovered regulator of the Arp2/3 complex that can inhibit nucleation and disassembly branches. We solved the structure of the 240-kDa assembly of Mus musculus GMF and Bos taurus Arp2/3 complex and found that GMF binds the barbed end of Arp2, overlapping with the proposed binding site of WASP-family proteins. The structure suggests that GMF can bind branch junctions in the manner that cofilin binds filament sides, consistent with a modified cofilin-like mechanism for debranching by GMF. The GMF-Arp2 interface reveals how the ADF-H actin-binding domain in GMF is exploited to specifically recognize Arp2/3 complex and not actin.

We set out to determine the structural bases for GMF function by solving the crystal structure of GMFγ (hereafter referred to as GMF) bound to the Arp2/3 complex. The structure revealed that GMF binds the end of Arp2, using a binding mode similar to that of other ADF-H domains with actin monomers. Our results showed how the ADF-H domain of GMF has evolved to bind Arp2 and not actin, providing the structural foundation for understanding how biochemical functions inherent to other ADF-H–domain proteins, such as filament severing, could be co-opted to operate at branch junctions instead of at filament sides. The structure also indicated that GMF may compete with the WASP C region for binding to Arp2, explaining how GMFs can inhibit nucleation by the complex. Finally, GMF binding caused ordering of subdomains 1 and 2 of Arp2, thus providing new structural insights into how Arp2 senses the γ-phosphate of ATP to influence the stability of branch junctions.

RESULTS

Crystal structure of GMF bound to the Arp2/3 complex

We cocrystallized bovine Arp2/3 complex with mouse GMFγ in the presence of ATP and calcium and collected X-ray diffraction data to 3.2-Å resolution. The data indexed as P65, with unit-cell lengths of 231.5 × 231.5 × 109.7 Å. We used the structure of ligand-free Arp2/3 complex as a starting model to solve the phases by molecular

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replacement (PDB 1TYQ)\(^9\). The structure showed clear electron density for all seven subunits of the Arp2/3 complex and one molecule of GMF, which contacts both the Arp2 and ARPC1 subunits (Fig. 1 and Supplementary Fig. 1). The refined model includes 2,013 of 2,123 total residues in the assembly, and we located side chains for all but 30 of the nonglycine residues. The final model had an \(R\)\(_{work}\) of 21.6% and an \(R\)\(_{free}\) of 24.1% (Table 1).

**GMF binds the Arp2 subunit**

Arp2 provides the major contact surface between GMF and the complex, burying 980 Å\(^2\) of its solvent-exposed surface area at the interface. This interaction occurs at the barbed end of Arp2 (Fig. 2). The mode of binding is similar to the interaction of ADF–H–domain proteins twinfilin and coflin with the barbed end of isolated actin monomers or with actin subunits in a filament, respectively\(^20,21\) (Fig. 2a,b). The similarities in these interfaces suggest that minor changes fine-tune the ADF–H domain of GMF to allow it to discriminate between the Arp2/3 complex and actin (described below). As with other ADF–H–domain proteins, the interaction with Arp2 can be broken into three regions of GMF: the N terminus, the \(\alpha\) helix and the \(\beta\)–\(\alpha\) loop (Fig. 2c). The N terminus adopts a different trajectory in Arp2/3-bound GMF than in free GMF\(^22\) (Supplementary Fig. 2), and this allows it to form a hydrophobic interface with \(\alpha\)L and the \(\alpha\)L–\(\alpha\)M loop on subdomain 1 of Arp2. This interface includes residues Val5 and Val7 in GMF and Leu361, Ile364 and Phe371 in Arp2. Hydrophobicity at the position of Val7 is maintained in other GMF sequences (Supplementary Fig. 3). Consistent with our structural observations, deletion of the first seven residues of GMF decreased binding to the Arp2/3 complex in a GST pulldown assay (Fig. 2d and Supplementary Fig. 4). In coflin, the N terminus has a critical role in mediating interactions with actin filaments, and deletion of the first five residues in budding-yeast coflin is lethal\(^23\). In addition, phosphorylation at Ser2 regulates coflin's activity by abolishing actin binding\(^24\). Previous reports have suggested that, similarly to the case with coflin, phosphorylation of GMF on a serine residue near the N terminus (Ser2) may regulate its activity\(^15\). Ser2 is disordered in the structure, and we did not find any obvious structural basis for an effect of phosphorylation of Ser2 on GMF activity.

The \(\beta\)–\(\alpha\) loop of GMF provides several polar contacts with Arp2. These include a hydrogen bond between Glu122 in GMF and His300 in Arp2 and salt bridges between Asp128 in GMF and Lys299 in Arp2 and between Lys137 in GMF and Glu296 in Arp2. Consistent with the importance of these contacts, mutation of Asp128 in GMF to lysine significantly decreased binding to the Arp2/3 complex (Fig. 2d). Arg124, a residue conserved in most GMF sequences (Supplementary Fig. 3), also inserts into the interface, making a hydrogen bond with the backbone of Gln149. This interaction is also important for binding the Arp2/3 complex (Fig. 2d) and may be critical for specifying recognition of Arp2 over actin, as discussed below. In contrast, mutation of Arg81, a residue not at the interface, did not significantly influence binding (Fig. 2d).

The long helix \(\alpha3\) in GMF forms the closest contact surface with Arp2, inserting into the front half of the hydrophobic groove between subdomains 1 and 3 in Arp2, referred to as the barbed-end groove. Met102, a residue conserved in GMF and other ADF–H–domain protein sequences, projects into the groove, contacting a hydrophobic face formed by Ile364, Val360, Leu361, Ala148 and Tyr147. These contacts are important for the interaction, as mutation of Met102 to alanine significantly decreased binding (Fig. 2d). In comparison to its position in free GMF, the \(\alpha3\) helix rotates slightly to position Met102 into the groove (Supplementary Fig. 2). The barbed-end groove in actin is a hot spot for interactions with regulatory proteins, and several proteins, including WASP, twinfilin and gelsolin, insert a hydrophobic face of a helix into the groove to bind the barbed end of actin\(^25\). That GMF uses the same mechanism for interaction with Arp2 indicates that the barbed-end groove of Arp2 may also be a hot spot for interaction of regulatory proteins.

**Contacts between GMF and ARPC1**

Bound GMF buries 380 Å\(^2\) of accessible surface area on ARPC1, contacting the outside (D) \(\beta\)–strand in \(\beta\)–propeller blade 3 (Fig. 3). Residues from the \(\alpha2\)–\(\beta3\) loop and the \(\beta4\)–\(\alpha3\) loop in GMF contribute to the interaction, with Glu63 and Gln65 from GMF forming...
GMF binding causes the ordering of subdomains 1 and 2 of Arp2. Comparisons to previously solved crystal structures of the Arp2/3 complex revealed that GMF binding did not change the overall position of the individual subunits in the complex. However, GMF binding caused subdomains 1 and 2 of Arp2 to become ordered, whereas in all previously solved crystal structures of the Arp2/3 complex, Arp2 is either partially or completely disordered. We were able to build all previously solved crystal structures of the Arp2/3 complex, Arp2 caused subdomains 1 and 2 of Arp2 to become ordered, whereas in the Trp131 and Lys97 in GMF. A comparison of ARPC1 sequences from diverse species revealed that residues in the β3D strand are well conserved. In contrast, most of the residues in GMF that contact ARPC1 are not conserved (Supplementary Fig. 3). Structural differences at the ARPC1-GMF interface may underlie potential differences in the influence of GMFs from different species on the Arp2/3 complex.

Conserved inserts in Arp3 may modulate GMF-Arp3 interaction Our structure suggests that GMF exerts its regulatory control over the Arp2/3 complex through interactions with Arp2. Like Arp2 and actin,
Arp3 has a hydrophobic barbed-end groove that could potentially interact with GMF. To determine how GMF preferentially binds Arp2, we overlaid Arp3 onto Arp2 in the GMF-bound-complex structure and examined the interface. Whereas many of the residues that contact GMF in Arp2 are conserved in Arp3, Arp3 has two critical regions that differ from Arp2. The first is an insertion within its actin core, the αD–β9 insert, which lengthens the αD–β9 loop and extends the αD helix by one turn (Fig. 5a). The extended αD helix and the αD–β9 loop both clash with GMF in the model. In addition to this potential steric clash, two key interfacial residues in αD at the GMF–Arp2 interface, Tyr147 and Gly150, are alanine and tryptophan, respectively, in Arp3. The second critical difference is the C terminus of Arp3, which is longer than that in Arp2 and actin and contains a phenylalanine residue (Phe414) not present in Arp2 or actin. This residue pins the C terminus into the hydrophobic barbed-end groove, where it would clash with α3 of GMF bound to the barbed-end groove (Fig. 5b). Although we cannot rule out the possibility that GMF binds Arp3 (ref. 18), binding would require conformational changes expected to weaken binding. Notably, the αD–β9 insert and the C-terminal extension are present in all Arp3 sequences we examined (Supplementary Fig. 5), thus suggesting that Arp2 may provide the primary interaction surface for GMF from diverse species.

**Molecular determinants of GMF specificity for Arp2 over actin**

GMF is the only one of five classes of ADF-H–domain proteins that does not bind actin16. To determine the structural basis for this molecular discrimination by GMF, we compared the GMF–Arp2 interface to the twinfilin C-terminal ADF-H domain (twinfilin–C)–actin interface20. Twinfilin is unusual among the ADF-H–domain proteins in that it contains tandem ADF-H domains. However, the C-terminal ADF-H domain, which is the only ADF-H domain crystallized with actin to date, binds both monomeric and filamentous actin and thus provides a good model for understanding actin–ADFH interactions20. Comparison of the structures of the two interfaces revealed that GMF specificity is achieved through matching of polar contacts at the interface and sliding of helix α3 in the barbed-end groove in the GMF-Arp2 interface, and this allows GMF to avoid clashing with ARPC1.

Matched polar contacts are evident in two key regions at the interface. Asp298 in the β5–α4 loop in twinfilin-C interacts with Arg147 in actin. GMF has an arginine (Arg124) in place of the aspartate, thus creating the potential for steric clash and electrostatic repulsion in a hypothetical GMF-actin interaction (Fig. 5c). Residues at the N terminus of α3 also appear to be critical for specificity. The basicity of Arg269 in twinfilin is conserved in twinfilins and cofilin, and this residue forms a salt bridge with Glu334 in actin20. This interaction is not possible in a modeled GMF-actin interaction, because Arg269 in twinfilin-C is replaced by a glutamine (Gln101) in GMF, and Glu334 in actin is replaced with an arginine (Arg349) in Arp2. Together, these interactions explain the specificity of GMF for Arp2 and also suggest how ADF-H–domain proteins other than GMF can selectively bind actin over Arp2.

The proximity of ARPC1 to Arp2 in the assembled complex provides an additional level of specificity for the GMF–Arp2 interaction. When we overlaid actin from the twinfilin-C–actin structure with Arp2, twinfilin clashed with residues from β-strand 3D in ARPC1. To avoid this clash, GMF slides back in the barbed-end groove, away from ARPC1 (Fig. 5d). Residues in the C-terminal end of the α3 helix specify this shifted binding register. In the twinfilin-C–actin
GMF may block binding of C to the barbed end of Arp2. A hypothetical model showing the C region of VCA (green) binding to the barbed end of Arp2 (red). Model was constructed by overlaying structure of V-bound actin (PDB 2A3Z) onto Arp2 from the GMF–Arp2/3 complex structure and threading the C sequence into V. GMF (pink) and Arp2 are shown as ribbons. ARPC1 is shown in surface representation with conserved residues colored blue. Numbers indicate conserved basic or hydrophobic residues that may contact the A region of VCA. Left, the approximate position of the mother filament on the basis of the branch-junction EM model is indicated with a gray bar with an arrow in the direction of the barbed end. Right, barbed end (gray semicircle) is pointed out of the page. Surfaces of ARPC1 that contact ARPC4 or ARPC5 are outlined with black dashed lines, and the approximate position of the ARPC5 N terminus is indicated by a yellow dashed line.

A model of GMF at the branch junction

Budding-yeast and mouse GMFs have been reported to disassemble Arp2/3-nucleated branches and 2 of subunit D2 (Figs. 5d and 7). This suggests that binding of GMF might cause a change in the Arp2-D2 interface that could destabilize the junction. Because cofilin does not ‘slide back’ in the groove of actin, this change is likely to be distinct from cofilin-induced changes to actin filaments. Unlike cofilin, which stimulates debranching indirectly though interactions with the mother filament of actin, GMF is thought to directly bind the Arp2/3 complex at filament junctions to disassemble branches. We modeled GMF bound at a branch junction to investigate how debranching might occur.

Supereposition of Arp2 from the GMF–Arp2/3 cocrystal structure onto the EM reconstruction of a branch junction revealed that the GMF-binding site is accessible in the assembled branch (Fig. 7). At the branch junction, GMF simultaneously contacts the barbed end of Arp2 and subdomains 1 and 2 of the adjacent actin monomer (subunit D2). The mode of binding is similar to the interaction of cofilin with adjacent subunits in an actin filament, and this suggests that GMF may use a cofilin-like mechanism to sever the daughter filament at the branch junction. Consistent with this observation, recent mutational analysis of budding-yeast GMF shows that residues in the ‘F-actin–binding’ region of GMF are important for debranching. However, key differences between cofilin and GMF point to potential mechanistic differences. First, sliding of GMF in the barbed-end groove of Arp2, described above, moves GMF toward actin subunit D2, creating a steric clash between residues in GMF with subdomains 1 and 2 of subunit D2 (Figs. 5d and 7). This suggests that binding of GMF might cause a change in the Arp2-D2 interface that could destabilize the junction. Because cofilin does not ‘slide back’ in the groove of actin, this change is likely to be distinct from cofilin-induced changes to actin filaments. Second, the region of GMF that contacts actin subunit D2, termed the F-actin–binding region, is structurally distinct in GMF. It includes a short antiparallel β-sheet between β3 and β4 and a short antiparallel β-sheet between β3 and β4 and is not present in other ADF-H proteins. This region contains several conserved residues specific to GMF, including Asp79, Arg81, Ser83 and Pro85 (Supplementary Fig. 3). The β3–β4 loop region in cofilin directly contacts actin filaments and has been shown to contribute to actin-filament binding but contains a distinct set of residues (Supplementary Fig. 3). GMF-specific residues at
Figure 7 Model of GMF bound to a branch junction. (a) Model of GMF (spheres) placed into EM branch-junction reconstruction by overlaying Arp2 from the GMF–Arp2/3 complex structure onto Arp2 from the EM branch-junction model38. Spheres in GMF are colored according to conservation (blue, most variable; cyan, variable; pink, high conservation; magenta, highest conservation)47. Actin subunits in the daughter filament are labeled D1 and D2. The approximate position of the mother filament is shaded gray, with the barbed end pointed out of the page. (b) Close-up of hypothetical model of GMF at a branch junction. Regions of clash are indicated with red arrowheads. The direction of sliding of GMF in the barbed-end groove is indicated with an arrow. Subdomains 1 and 2 of actin subunit D2 and β-propeller 3 of ARPC1 are indicated. (c) Same as a but rotated to show surface of GMF in contact with Arp2 and actin subunit D2. (d) Ribbon diagram of GMF showing most-conserved residues in GMF sequences (magenta) and approximate regions of contact with Arp2 and subunit D2 (F-actin–binding region). The orientation of GMF in this panel is the same as in c.

the end of helix α1 and in the α1–β1 loop also contact subunit D2 in the branch model. Although this segment has not been mutationally probed in cofilin, it makes close contacts with the filament in an EM reconstruction of cofilin-bound filaments21. Together, these observations support a modified cofilin-like mechanism for GMF-mediated disassembly of branch junctions.

GMF may block actin-monomer recruitment during activation

Comparison of the GMF-bound complex to the EM reconstruction of a branch junction revealed a potential structural impediment to activation when GMF is bound38. As noted above, GMF at the branch junction clashes with the actin monomer (D2) bound to the barbed end of Arp2 (Fig. 7). This suggests that GMF may block actin-monomer recruitment by the actin monomer recruited by VCA to the barbed end of Arp2 during activation39, thus providing an additional level of regulation of the complex.

DISCUSSION

Diverse classes of Arp2/3-complex inhibitors target distinct steps in the branching nucleation pathway. Inhibitors such as coronin5, tropomyosin8, caldesmon11 and EPLIN7 exploit the requirement of the Arp2/3 complex to bind preexisting filaments to downregulate the nucleation reaction. These proteins bind actin filaments to block Arp2/3 complex–binding sites, thereby indirectly inhibiting the complex. Other inhibitors, such as the PDZ-BAR-domain protein Pick1, contain acidic regions that mimic the A region of VCA and directly compete with VCA for binding to the complex10. GMF may use a similar mechanism to block activator binding, targeting the C-binding site instead of A. However, a simple competition mechanism cannot fully explain the inhibition, because GMF binding is unlikely to block A-binding sites on ARPC1. We hypothesize that by displacing VCA from the C-binding site and sliding back in the barbed end groove, GMF may prevent proper positioning of VCA-recruited actin monomers at the barbed end of Arp2. Finally, we note that some Arp2/3-complex regulators, including small-molecule inhibitors CK-666 and CK-869, have been shown to directly target the activating conformational change in the Arp2/3 complex stimulated by VCA and actin monomers30. Averaged single-particle EM images suggest that GMF may block the movement of the complex into an activated conformation18. Although our structure does not provide an obvious mechanism by which GMF could block this step, without higher-resolution structures of the activated state we cannot rule out this possibility.

Our data show that GMF overlaps with the proposed C site on Arp2 but does not bind the proposed C site on Arp3. The ability of GMF to preferentially target Arp2 may have an important role in defining its influence on the complex and is consistent with the conserved structural features on Arp3 that may hinder GMF binding. Another Arp2/3 regulator, cortactin, specifically binds Arp3 where it competes with VCA40. However, instead of inhibiting WASP-induced activation of the complex, cortactin synergizes with WASP to markedly increase nucleation41. Determining precisely how the binding mode of a regulator influences its ability to modulate Arp2/3 activity will be critical for understanding not only the structural mechanism of activation but also the complex interplay between regulators in vitro and in vivo.

Arp2/3-nucleated branches dissociate on the timescale of minutes in vitro, but Arp2/3-mediated networks turn over in seconds in vivo42. Debranching contributes to turnover in yeast actin patches and lamellipodia28 and may have a general role in remodeling dynamic actin networks. GMF, coronin1B and cofilin, three proteins reported to have debranching activity, each have a distinct mechanism that probably defines their function in a cellular context37,43. Our data suggest that GMF targets branch junctions at the interface between Arp2 and the daughter filament, whereas cofilin and coronin1B bind actin filaments or both actin filaments and the Arp2/3 complex, respectively. These distinctions will influence how effectively each debrancher competes with actin-binding proteins that target and stabilize branch junctions, such as cortactin, or filament sides, such as tropomyosin44,45. Understanding how the debranching activity of GMF and other debranchers is influenced by the cellular milieu of actin-binding proteins will be critical for understanding how actin-filament networks are turned over in vivo.
Methods

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structural factors have been deposited in the Protein Data Bank, under accession code 4JD2.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Author Contributions

Q.L and B.J.N. designed the research; Q.L performed all experiments; Q.L and B.J.N. analyzed the data and wrote the paper.

Competing Financial Interests

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. We purified *B. taurus* Arp2/3 complex as previously described\(^\text{46}\). Mouse GMF\(\gamma\) was subcloned from a plasmid provided by B. Goode into the pGV67 (ref. \(^48\)), which tags the N terminus with glutathione S-transferase and a TEV-protease tagging site. Point mutations and truncations of GMF\(\gamma\) were made in the context of the pGV67 plasmid. BL21(DE3)RIIL *E. coli* transformed with GMF in pGV67 were grown to an OD\(_{600}\) of 0.6 before addition of 0.4 mM IPTG and expression at 22 °C overnight. Cells were harvested and lysed by sonication. Clarified lysate was loaded onto a glutathione Sepharose 4B glutathione-affinity column (GE Healthcare), washed with binding buffer (20 mM Tris, pH 8.0, 140 mM NaCl, 2 mM EDTA and 1 mM DTT) and eluted with binding buffer containing 50 mM reduced glutathione. TEV protease was added to pooled fractions, and the sample was dialyzed overnight against 10 mM CHES, pH 9.5, 25 mM NaCl and 1 mM DTT. The dialyzed sample was then loaded onto a 6-ml Resource Q column (GE Healthcare) and eluted with a 25-mM to 500-mM gradient. Peak fractions were pooled, concentrated and purified on a Superdex 75 gel-filtration column (GE Healthcare) in 20 mM Tris, pH 8.0, 100 mM NaCl and 1 mM DTT.

GST pulldown assays. GST-GMF at 60 µM was bound to glutathione Sepharose beads and incubated with 1 µM *B. taurus* Arp2/3 complex in 50 mM KCl, 10 mM imidazole, pH 7.0, 1 mM EGTA, 1 mM MgCl\(_2\), 0.2 mM adenosine diphosphate (ADP) and 1 mM dithiothreitol for 1 h at 4 °C. Samples were spun, and both supernatant and washed pellet were loaded on SDS-PAGE gels. Arp2/3 complex in supernatant and pellet was visualized by blotting with an anti-ARPC2 antibody (Millipore, 07-227) diluted 1:1,000 and a donkey anti-rabbit IgG-HRP antibody (Santa Cruz, sc2313) diluted 1:10,000. The fraction bound was measured with Li-Cor imaging software.

Crystal growth, data collection and refinement. A solution containing 25 µM Arp2/3 complex, 25 µM GMF\(\gamma\) in 50 mM Tris, pH 8.0, 500 mM ATP, 500 µM CaCl\(_2\) and 1 mM DTT was mixed 1:1 with 10.6% polyethylene glycol 400 and allowed to equilibrate by vapor diffusion from a hanging drop at 4 °C. Crystals grew to ~50 × 50 × 100 µm in ~10 d. Crystals were cryoprotected by direct addition of a solution of 50 mM Tris, pH 8.0, 50% PEG 400, 500 µM ATP and 500 µM CaCl\(_2\) and flash frozen in liquid nitrogen. Data were collected at 100 K at a wavelength of 0.9793 Å at beamline 19-ID at Argonne National Laboratory and processed with HKL2000 (ref. \(^49\)). A molecular replacement solution was found with Phaser\(^50\), with the ATP-bound-structure of BtArp2/3 complex as a search model (1TYQ\(^\text{19}\)). Refinement was initiated by rigid body minimization in CCP4 (ref. \(^51\)), allowing each subunit of the complex, plus subdomains 1 and 2 of Arp3 to move independently. Minimization was continued in Refmac, using tight geometry constraints (weighting set at 0.002), jelly body refinement (\(\sigma\) at 0.01) and TLS refinement. Density for GMF was clearly visible even in the first electron density maps but was not added until the second round of refinement. Subdomains 1 and 2 of Arp2 were built piecemeal, starting in the second round of refinement. The Ramachandran statistics for the final refined structure are: most favored residues, 1,581 (89.0%); additionally allowed regions, 195 (11.0%); generously allowed, 0 (0%); disallowed 0 (0%).

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