Dissecting Requirements for Auto-inhibition of Actin Nucleation by the Formin, mDia1*

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The mammalian formin, mDia1, is an actin nucleation factor. Experiments in cells and in vitro show that the N-terminal region potently inhibits nucleation by the formin homology 2 (FH2) domain-containing C terminus and that RhoA binding to the N terminus partially relieves this inhibition. Cellular experiments suggest that potent inhibition depends upon the presence of the diaphanous auto-regulatory domain (DAD) C-terminal to FH2. In this study, we examine in detail the N-terminal and C-terminal regions required for this inhibition and for RhoA relief. Limited proteolysis of an N-terminal construct from residues 1–548 identifies two stable truncations: 129–548 and 129–369. Analytical ultracentrifugation suggests that 1–548 and 129–548 are dimers, whereas 129–369 is monomeric. All three N-terminal constructs inhibit nucleation by the full C terminus. Although inhibition by 1–548 is partially relieved by RhoA, inhibition by 129–548 or 129–369 is RhoA-resistant. At the C terminus, DAD deletion does not affect nucleation but decreases inhibitory potency of 1–548 by 20,000-fold. Synthetic DAD peptide binds both 1–548 and 129–548 with similar affinity and partially relieves nucleation inhibition. C-terminal constructs are stable dimers. Our conclusions are as follows: 1) DAD is an affinity-enhancing motif for auto-inhibition; 2) an N-terminal domain spanning residues 129–369 (called DID for diaphanous inhibitory domain) is sufficient for auto-inhibition; 3) a dimerization region C-terminal to DID increases the inhibitory ability of DID; and 4) DID alone is not sufficient for RhoA relief of auto-inhibition, suggesting that sequences N-terminal to DID are important to RhoA binding. An additional finding is that FH2 domain-containing constructs of mDia1 and mDia2 lose >75% nucleation activity upon freeze-thaw.

Formin proteins are emerging as regulators of many cellular actin-based structures (1, 2). Biochemically, formins exert several effects on actin polymerization dynamics, including acceleration of filament nucleation from monomers, inhibition of barbed end elongation rate, inhibition of complete barbed end capping by heterodimeric capping protein, and filament severing (3–10). These in vitro activities are generally considered to result from the ability of formins to bind at or near the filament barbed end and to move processively with the barbed end as it elongates (11, 12). Essential to these properties is the formin homology 2 (FH2) domain, a 400-residue region generally found in the C-terminal half of the protein. Biochemical and structural studies show that the FH2 domain is dimeric for several formins (10, 13, 14), although longer constructs of the budding yeast formin, Bni1p, can tetramerize (6).

Mammals possess 15 formin genes, in seven distinct phylogenetic groups (15). For one mammalian formin, mDia1, the mechanisms regulating effects on actin have begun to be elucidated. The in vitro nucleation activity of the FH2-containing C terminus of mDia1 is inhibited potently by inclusion of a separate polypeptide containing the mDia1 N terminus (9), suggesting an auto-inhibitory regulatory mechanism. Cellular experiments and two-hybrid interactions implicate a short sequence C-terminal to FH2, known as the diaphanous auto-regulatory domain (DAD), as a critical binding site for the N terminus in both mDia1 and the related protein, mDia2 (16). Direct effects of DAD on auto-inhibition of actin nucleation in vitro have not been examined.

Cellular studies suggest that binding of the Rho family GTPase, RhoA, to the N terminus of mDia1 can relieve auto-inhibition (17). Biochemical studies on actin nucleation support these findings (9), with the caveat that RhoA does not relieve completely the auto-inhibitory effect of the N terminus of mDia1. This incomplete relief by RhoA might imply that a second, non-RhoA dependent, auto-inhibitory interaction between the N and C terminus might exist.

In this study, we examine mDia1 auto-inhibition in more detail. Deletion experiments show that auto-inhibition can be uncoupled from RhoA relief, suggesting that mDia1’s N-terminal binding sites for RhoA and for the mDia1 C terminus are not identical. In addition, deletion of DAD from the C terminus does not affect nucleation but decreases the inhibitory potency of the N terminus 20,000-fold, suggesting that DAD mediates a high affinity interaction important for potent auto-inhibition. A synthetic DAD peptide binds the N terminus and partially disrupts auto-inhibition, supporting the role of DAD.

EXPERIMENTAL PROCEDURES

DNA Constructs—Constructs of mouse mDia1 (accession number U99863) were generated by reverse transcription-PCR and cloned into pGEX-KT, as described previously (9). Deletion constructs were generated by PCR from longer constructs, using Pfu DNA polymerase (Stratagene). The mouse mDia2 521–1171 construct in pGEX-KT was a kind gift from Dr. Arthur Alberts (Van Andel Research Institute).

Protein Preparation and Purification—All proteins were expressed and purified through the thrombin cleavage step following the procedure described in detail in Refs. 9 and 10. After elution of thrombin-cleaved protein from glutathione-Sepharose, further purification varied as follows. N-terminal constructs (1–548, 129–548, and 129–369) were purified by fast protein liquid chromatography on a SourceS15 5/5 or 10/10 column (Amersham Biosciences) and then concentrated on Q Sepharose Fast Flow (Amersham Biosciences) and dialyzed into the auto-regulatory domain; DID, diaphanous inhibitory domain; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GMP-PNP, guanosine 5’-β,γ-imido)triphosphate; GDP, guanosine 5’-diphosphate.

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1 The abbreviations used are: FH, formin homology; DAD, diaphanous

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following buffer: 2 mM NaPO₄, pH 7.0, 50 mM NaCl, 0.1 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT. N-terminal constructs could be frozen in aliquots of <50 μl in liquid nitrogen and subsequently stored at −70 °C with no loss of inhibitory activity or detectable aggregation (10). C-terminal constructs (748–1255, 748–1203, 748–1175, and mDia2–512–1171)) were purified by fast protein liquid chromatography on a SourceQ15/5 or 10/10 column (Amersham Biosciences), concentrated on SP Sephrose Fast Flow (Amersham Biosciences), and dialyzed into the following buffer: 2 mM NaPO₄, pH 7.0, 150 mM NaCl, 0.1 mM EGTA, 0.5 mM DTT. C-terminal constructs lost >75% of their nucleation activity when frozen. C-terminal constructs were stored at concentrations <10 μM at 4 °C for 2 weeks or with 1 volume of glycerol at −20 °C for two months with no loss of activity. Protein concentrations were determined by two methods: from absorbance at 280 nm (extinction coefficient calculated by protein sequence using expasy.org/cgi-bin/protparam) and by Coomassie-stained SDS-PAGE using known amounts of actin as standards. Rabbit skeletal muscle actin obtained from acetic acid powder (USB) and labeled with pyrenyl iodacetamide (19). Both unlabeled and labeled actin were gel-filtered on S200 (20), which was crucial to obtain reproducible polymerization kinetics.

**DAD Peptide Synthesis, Fluorescent Labeling, and Fluorescence Anisotropy—**A peptide containing amino acids 117–120 of mouse mDia1 (DTEGVMDSILLEALQSGAAFRKRRK) was synthesized by the W. M. Keck Biotechnology Resource Laboratory, using 10 nM FITC-DAD in 10 mM imidazole, pH 7.0, 50 mM EGTA, and 0.5 mM DTT. Protein concentrations were confirmed by matrix-assisted laser desorption ionization.

**Actin Polymerization by Fluorescence Spectroscopy—**A detailed procedure is described in Ref. 22. Unlabeled and pyrene-labeled actin were mixed in G buffer (2 mM Tris, pH 8.0, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl₂, and 0.01% NaN₃) to produce an actin stock of the desired pyrene-labeled actin concentration (5%). Actin polymerization was initiated by the addition of CaCl₂, and fluorescence of fully polymerized and unpolymerized actin was measured in a PC1 spectrofluorimeter (ISS Inc.) or an LS50B spectrofluorimeter (PerkinElmer Life Sciences). The time between the mixing of final components and start of fluorescence data collection was measured for each assay and ranged between 10 and 25 s.

**Calculating Filament Concentration—**Slopes of pyrene fluorescence from polymerization time courses were determined at the 50% point of polymerization with Kaleidagraph (Synergy Software, Reading, PA). Slopes of pyrene fluorescence were fit to a single species model. For mDia1 C-terminal fragments, this absorbance was equivalent to 1 mM MgCl₂ immediately prior to polymerization. Polymerization was induced by the addition of 10× KMEI (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, and 100 mM imidazole, pH 7.0) to a concentration of 1×, with the remaining volume made up by G buffer. Added proteins were mixed together for 1 min prior to their rapid addition to actin to start the assay. Pyrene fluorescence (excitation 375 nm, emission 407 nm) was monitored in a PC1 spectrofluorimeter (ISS Inc.) or an LS50B spectrofluorimeter (PerkinElmer Life Sciences). The time between the mixing of final components and start of fluorescence data collection was measured for each assay and ranged between 10 and 25 s.

**Results**

**Physical Properties of mDia1 N-terminal Constructs—**We have shown previously that an N-terminal fragment of mDia1 (residues 1–548, Fig. 1A) partially inhibits actin nucleation by the FH2 domain-containing C-terminal construct (9). In this study, we further dissected the N terminus. First, we characterized two proteolytically derived deletions of 1–548: a chymotrypsin-generated fragment from residues 120–548 and a proteinase K-generated fragment from residues 129–369 (Fig. 1A). For proteinase K, digestion at 20 °C for up to 60 min resulted in no additional cleavage products (not shown). Given the promiscuous nature of proteinase K (24, 25), resistance to digestion under these conditions suggests that the region from 129 to 369 is very stably folded. For chymotrypsin, digestion at 4 °C for 1 h produced the 120–548 fragment, whereas digestion at 20 °C produced an additional product with similar SDS-
Effects of N-terminal Deletions on Actin Nucleation—We characterized the effects of 1–548, 129–548, and 129–369 on actin polymerization kinetics using the pyrene-actin polymerization assay in the absence or presence of 2.5 nm mDia1 748–1203 (Fig. 1A). This construct contains the FH2 domain and DAD and is a potent nucleator (Fig. 3). None of the N-terminal constructs alone affected actin polymerization kinetics at concentrations up to 20 μM (9) and not shown). In contrast, all N-terminal constructs inhibited nucleation by 748–1203 (Fig. 3A, A–C). The IC50 values for each construct were ~2 nM for 1–548, 20 nM for 129–548, and 200 nM for 129–369 (Fig. 3D). When 748–1255 was used as the nucleation factor, results were indistinguishable from those using 748–1203 (not shown).

We then tested the ability of RhoA to relieve inhibition by the N-terminal constructs. In contrast to 1–548, the inhibition of which is partially relieved by RhoA (9), the inhibitory activities of 129–548 and 129–369 are unaffected by high concentrations of RhoA (Fig. 4 and not shown). The result was identical with either GDP or GMP-PNP-bound RhoA.

Importance of DAD to Auto-inhibition—From the data in Fig. 3, a construct containing the FH2 domain and DAD (748–1203) behaves similarly to a construct containing the entire C terminus (748–1255 (9)) in that it is a potent nucleator and its nucleation ability is inhibited by N-terminal constructs. To determine the role of DAD in auto-inhibition, we expressed a shorter construct in which DAD was truncated (748–1175, Fig. 1A). This construct was equally potent to 748–1255 and 748–1203 in actin nucleation (Fig. 5A). We next compared the ability of 748–1175 to be inhibited by 1–548. Measurable inhibition required 1–548 concentrations >10 μM (Fig. 5B), but complete inhibition was never attained at the concentrations tested (Fig. 5C). The estimated IC50 of 1–548 for 748–1175 is 40 μM.

To test directly an interaction between DAD and the N-terminus, we synthesized a fluorescently-coupled 25-residue peptide containing the DAD sequence (FITC-DAD). By fluorescence anisotropy (26), FITC-DAD binds both 1–548 and 129–548 (Fig. 6A), with a Kd of 0.25 μM. The anisotropy change produced by 129–369 was too small to determine affinity with accuracy, but the presence of 129–369 consistently raised the anisotropy of FITC-DAD, suggesting that it bound as well. FITC-DAD was sufficient to disrupt the auto-inhibitory interaction between 748–1255 and 1–548 (Fig. 6B). Similar to RhoA relief of auto-inhibition, FITC-DAD was unable to cause full recovery of 748–1203 activity (Fig. 6C).

Effect of Freezing on mDia1 Nucleation Activity—Several laboratories have published work in which nucleation by mDia1 FH2 domain-containing constructs is examined (7, 9, 14, 27, 28), and the nucleation potency of mDia1 varies widely among these studies. Since this variability could influence mechanistic interpretations, we sought to determine factors that might cause these activity changes. We found that “flash” freezing resulted in the loss of >75% nucleation potency for
mDia1 748–1203 and mDia2 521–1171 (containing FH1, FH2, DAD, and C-terminal sequences), as well as for mDia1 748–1255 and 748–1175 (Fig. 7 and not shown). Samples were frozen in 10-\%/H2O2 aliquots in thin-walled PCR tubes by plunging into liquid nitrogen, conditions in which the sample was frozen in <2 s. This loss of activity occurred regardless of the presence of 50% glycerol. In contrast, similar freezing of mDia1 N-terminal constructs did not affect their abilities to inhibit nucleation (not shown).

**DISCUSSION**

In this study, we characterize the auto-inhibitory properties of mDia1 biochemically, with the following results. First, our work extends previous cellular findings of DAD function (16), suggesting that the role of DAD is to increase binding affinity to the N-terminal auto-inhibitory domains rather than to be part of the auto-inhibitory mechanism. Second, we identify a core inhibitory region, comprising residues 129–369, which we refer to as DID (diaphanous inhibitory domain). Third, we show that auto-inhibition can be uncoupled from RhoA relief of auto-inhibition since inhibition by the minimal DID or DID with a C-terminal extension is not relievable by RhoA. Fourth, we identify a region between DID and the FH1 domain that mediates dimerization.

We identify DID by limited proteolysis using proteinase K. The exceptional resistance of residues 129–369 to proteinase K suggests that this region adopts a stable tertiary structure.
tion, the ability of this region to inhibit nucleation by the mDia1 C terminus suggests that it mediates the major auto-inhibitory interaction. We predict that this region contains the DAD-interacting region. Sequence alignments demonstrate that many formins, including members of the metazoan DAAM and FRL groups, as well as budding yeast Bni1p, contain regions similar to DID (15). All of these proteins also possess putative DADs. Thus, auto-inhibitory regulation through DID/DAD binding may be common to many formins. Notable exceptions are proteins in the delphilin, FMN, and INF metazoan groups (15), which we predict to be regulated by different mechanisms.

In contrast, since neither this region nor the 129–548 construct appears to interact productively with RhoA, motifs important for RhoA binding must lie N-terminal to residue 129. Previous two-hybrid studies found that RhoA bound an mDia1 construct containing residues 63–260 (29). Possibly, full affinity for RhoA requires residues both within DID and N-terminal to DID.

Inclusion of residues from 369 to 548 causes N-terminal fragments to dimerize with affinity in the 100 nM range. Since a coiled-coil region is strongly predicted from 470 to 550 (15), we hypothesize that this coiled-coil region mediates dimerization, raising at least two questions. First, does this region dimerize in a parallel or antiparallel orientation? Second, what is the multimeric state of full-length mDia1, which contains both this dimerization region and the dimeric FH2 domain? Answers to these questions will be necessary for a complete understanding of mDia1 regulation.

Our experiments clearly show that DAD is required for high affinity auto-inhibition. Deletion of DAD from the C terminus raises the IC50 of the N-terminal 1–548 construct from 2 nM to 40 μM, or 20,000-fold. Synthetic DAD peptide binds DID-containing constructs and partially relieves inhibition. Mutations that disrupt DAD function in cells also disrupt DAD function in our biochemical assays.4

Since DAD is not required for nucleation, and inhibition of the DAD-less C terminus can be affected at high concentrations of N terminus, we hypothesize that the role of DAD is to supply a high affinity interaction that enables a second interaction between DID and the FH2 domain. This second interaction is inhibitory to nucleation. The fact that RhoA does not fully relieve auto-inhibition suggests that a second activator is required for full activation. This activator may disrupt the non-DAD-mediated interaction or further destabilize the DID/DAD interaction.

The nucleation activity of mDia1 and mDia2 FH2 domain-containing constructs is strongly diminished by freezing. This fact might contribute to varying mDia1 nucleation potencies observed by different laboratories (7, 9, 14, 27, 28). The sensi-

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tivity to freezing might imply that FH2 domain-containing constructs are sensitive to other storage conditions as well. We observe that mDia1 FH2 domain-containing constructs partially precipitate in a variety of buffer conditions when stored at high concentrations \((10^3 \text{M})\) at 4 °C for extended periods (3 days). For these reasons, we store mDia1 \((10^3 \text{M})\) at 4 °C, in which case full activity is maintained for 2 weeks. For longer storage, the addition of glycerol to 50% v/v and storage at 20 °C maintains full activity for several months. This sensitivity to freezing might not be universal for FH2 domains since freezing FH2 domain-containing constructs of FRL1 (10) does not affect polymerization or severing activities.5

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