The Basic Region of the Diaphanous-autoregulatory Domain (DAD) Is Required for Autoregulatory Interactions with the Diaphanous-related Formin Inhibitory Domain

Mammalian diaphanous-related (mDia) formins act as Rho GTPase effectors during cytoskeletal remodeling. Rho binding to mDia amino-terminal GTPase-binding domains (GBDs) causes the adjacent Dia-inhibitory domain (DID) to release the carboxy-terminal Dia-autoregulatory (DAD) domain that flanks the formin homology-2 (FH2) domain. The release of DAD allows the FH2 domain to then nucleate and elongate nonbranched actin filaments.

DAD, initially discovered as a region of homology shared between a phylogenetically divergent set of formin proteins, is comprised of a core motif, MDXXLXL, and an adjacent region comprised of numerous basic residues, typically RRKR in the mDia family. Here, we show that these specific amino acids within the basic region of DAD contribute to the binding of DID and therefore the maintenance of the mDia autoregulatory mechanism. In addition, expression of full-length versions of mDia2 containing amino acid substitutions in either the DAD core or basic regions causes profound changes in the F-actin architecture, including the formation of filopodia-like structures that rapidly elongate from the cell edge. These studies further refine our understanding of the molecular contribution of DAD to mDia control and the role of mDia2 in the assembly of membrane protrusions.

Formins participate in the assembly of both the actin and microtubule cytoskeletons in multiple contexts, including cell division, migration, and development (1). In common with all formins, the mammalian Diaphanous-related formins, mDia1, mDia2, and mDia3 (2–5), have a formin homology-2 (FH2) domain that has multiple activities in vitro, including the nucleation of actin filaments from monomers, competition with barbed-end capping proteins, filament severing, and progressive elongation by moving on the barbed end of filaments (6–13), as recently reviewed by Higgs (14). The FH2 domain also serves as a dimerization interface (15, 16). mDia1 and mDia2 also associate with microtubule end-binding proteins and affect microtubule dynamics (17, 18).

Rho GTPase binding to the GTPase-binding domain (GBD) is thought to activate mDia proteins by disrupting an intramolecular autoinhibitory mechanism mediated by an amino-terminal diaphanous-inhibitory domain (DID) and the diaphanous autoregulatory domain (DAD) in the carboxyl terminus (20). Autoinhibition was recently shown to be mediated by DID, which is sufficient to weakly bind and inhibit nucleation by the isolated FH2 domain (12). Structure determinations have shown that DID is primarily a helical region with Armadillo repeats (21, 22). High-affinity DID binding and inhibition is bolstered by the presence of DAD (FH2-DAD), which increases the potency of inhibition by ~20,000-fold. This suggests that the role of DAD is to act as a high-affinity anchor for the DID region, thereby allowing the DID-DAD interaction to keep the mDia protein inactive until signaled by a small GTPase.

DAD was originally described by the consensus sequence shared between a subset of diaphanous-related formins that included a “core” motif (GA)(VA)MDXXLXL(KRQ)(GA)(SGA)(AP) (20). An additional “basic region,” RRKR in the mDia family, was also identified. As the formin family has expanded, additional comparisons suggest that the “DAD core,” MDXXLXL, is highly conserved (14). In the initial study, alanine-scanning replacements of conserved or similar residues within the core (Fig. 1A) demonstrated that residues such as methionine 1041 in mDia2 were critical for in vitro binding to the GBD/DID region in the amino terminus (20). These amino acid substitutions also interfered with the ability of an expressed EGFP-DAD fusion to trigger various cellular events such as: 1) the formation of thin actin filaments and 2) gene expression controlled by the serum response transcription factor (3, 23, 24) stabilization of microtubules (17).

The effects of DAD expression are similar to the effects of deregulated (activated) versions of mDia proteins lacking all or part of the autoinhibitory GBD/DID regions (19, 25, 26). Whereas DAD does not appear to have any inherent ability to affect actin nucleation (12), the effects of DAD expression were shown to be dependent upon the cellular mDia protein (20). In support of this model, a DAD peptide has been shown to activate autoinhibited mDia1 in vitro (12). Furthermore, DAD has been shown to trigger formin-dependent actin remodeling in budding yeast (27).

Whereas specific amino acid residues in the core region of DAD have been studied for their role in the autoregulation of full-length mDia, the adjacent basic region has yet to be examined in detail. In this study, we have investigated the molecular requirements of DAD for binding to...
DID and show that, in addition to the core region, specific basic residues in DAD are also necessary for binding to DID.

MATERIALS AND METHODS

Synthesis of DAD Peptides and Expression and Purification of GST Fusion Constructs—All of the fluorescent peptides containing the DAD region were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. All peptides were modified with 5,6-carboxyfluorescein at the NH$_2$ terminus and amidated on the COOH terminus. Each peptide consisted of residues 1036–1061 (wild type, DETGVMDSSLLEALQGAFFDRRRKRT). The mutant DAD peptides were created separately with the inclusion of the appropriate substituted amino acid(s).

For expression of GST fusion proteins, Rosetta (DE3) cells (Novagen) containing pGex-KT fusion constructs were grown in LB (100 µg/ml ampicillin, 50 µg/ml chloramphenicol) at 37 °C, induced at an $A_{600}$ of 0.6–0.8 with 0.5 mM isopropyl β-D-thiogalactopyranoside, and incubated overnight at 16 °C. The cells were harvested by centrifugation at 8,000 × g for 10 min at 4 °C, and then resuspended and sonicated in lysis buffer: TNM buffer (25 mM Tris-Cl, pH 7.0, 100 mM NaCl, and 10 mM MgCl$_2$) containing HALT protease inhibitors (Pierce), 20 units of DNase I, and 5 mM dithiothreitol. After centrifugation at 15,000 × g for 30 min at 4 °C, the supernatant fraction was loaded onto a glutathione-agarose column (Pierce) equilibrated with TNM buffer. The column was washed three times with TNM buffer, followed by elution of the GST fusion protein by the addition of 100 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione, and 10 mM dithiothreitol. The eluted protein was concentrated using an Amicon Ultra-15 centrifugal concentrator (Millipore). To remove the vast majority of the dithiothreitol and reduced glutathione from the protein sample, as well as restore the pH to 7.0, the concentrated protein sample was re-diluted with TNM buffer followed by concentration on the sample; this process was repeated three times.

Protein concentrations were determined by two methods: by measuring absorbance at 280 nm and by the Bio-Rad protein assay kit (using bovine serum albumin as the standard).

Fluorescence Anisotropy and Data Analysis (DAD-DID Binding)—All fluorescent measurements were taken at 25 °C on a Photon Technologies Inc. model QM-7/2003 SE fluorometer with polarization filters. For the anisotropy experiments, the anisotropy experiments, the fluorescence microscope equipped with a ×100 (1.4NA) objective using fixed acquisition times. F-actin levels were quantified by determining the mean fluorescent intensity of TRITC-phalloidin staining using image quantification software as previously described elsewhere (29); fluorescein isothiocyanate or EGFP fluorescent images were used to differentiate microinjected/expressing cells from un.injected cells.

Time-lapse image acquisition was performed on a Zeiss 100 inverted microscope equipped with an environmental chamber. Cells were microinjected with expression plasmid and image acquisition began 4 h later. Frames were acquired at 3-s intervals and the Quicktime movie was assembled using the Improvision Openlab software.

Plasmids and GenBank® Accession Numbers—mDia2 and various domain expression constructs were made in either pEF$_{myc}$ (His$_6$ tag/myc tag) or pEF$_{m}$ (myc tag), pEF$_{m}$-EGFP, using standard methods; details are available upon request. pEYFP-mDia2-M1041A was generated using pEYFP-mDia2 (30) as a template; the QuikChange® (Stratagene) site-directed mutagenesis was used to generate specific amino acid substitutions as per the manufacturer’s instructions; all plasmids were sequenced to confirm mutagenesis. The accession number for mDia2 is AF094519.

RESULTS

The Basic Region of DAD Is Required for DID Binding—DAD is located in the extreme carboxyl termini of the mDia proteins adjacent to the FH2 domain (12, 14, 20, 21). The methionine residue at position 1041 (Met$^{1041}$) in mDia2 has previously been shown by yeast two-hybrid and GST “pull down” assay to be critical for in vitro binding to the GBD/DID region in the amino terminus (20). In addition to the conserved Met$^{1041}$, most diaphanous-related formins contain a stretch of basic residues on the COOH-terminal side of the core DAD domain (Fig. 1, A and B). To assess the importance of the basic region of DAD, we first tested the ability of a DAD construct lacking this region to induce actin remodeling in cells.

EGFP-DAD expression in NIH 3T3 cells causes the accumulation of numerous thin, parallel actin filaments that traverse the cell, stabilize microtubules, and activates the serum response transcription factor (17, 20). As shown in Fig. 1, C–H, EGFP-DAD (or its variants) was expressed for 4 h after microinjection of the expression plasmid in NIH 3T3 cells previously maintained overnight in low serum (0.1% fetal calf serum). After fixation, cells were stained with TRITC-phalloidin to visualize F-actin assembly, as shown in the right-hand panels; EGFP-DAD fusion proteins are shown in the left-hand panels. EGFP-DAD expression (Fig. 1, C and D) caused the formation of stress fiber-like actin filaments that traverse the cell. Similar to previous work, EGFP-DAD M1041A (Fig. 1, E and F) did not affect the actin architecture relative to non-expressing cells or cells expressing EGFP alone (data not shown). In addition, a version of EGFP-DAD ending at Phe$^{1052}$ (lacking any basic region) also...
**Molecular Requirements for DAD-DID Binding**

**A**

| p140 mDia1 | 1177 |
| p134 mDia2 | 1036 |
| p100 mDia3 | 1051 |

**B**

GMD: DIA-automotory domain; DID: DIA-inhibitory domain; DD: dimerization domain; CC: coiled-coil; FH1: formin homology-1; FH2: formin homology-2; D (DAD),

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**FIGURE 1. mDia2 is an autoregulated multidomain formin.** A, domain structure of p134 mDia2. GBD, GTPase-binding domain; DID, DIA-inhibitory domain; DD, dimerization domain; CC, coiled-coil; FH1, formin homology-1; FH2, formin homology-2; D (DAD),

had no effect on the actin architecture. EGFP-DAD (but not the M1041A or R1055Stop variants) increased the overall levels of F-actin in cells as shown by quantitating the fluorescence intensity of TRITC-phalloidin staining in expressing cells (Fig. 1I) using previously described methods (29).

To further investigate the specific amino acid residues in the basic region that may be important to the ability of DAD to affect cellular actin remodeling, a series of double mutants were tested in microinjection experiments. As shown in Fig. 2, A–H, the DAD changes in the basic region that failed to stimulate actin remodeling were the double glutamate versions, EGFP-DAD R1057/R1058E (Fig. 2, C and D) and K1059/R1060E (Fig. 2, E and F). Glutamates substituted at the nonconserved basic residues Lys1063 and Lys1065 had no effect on DAD-induced actin filament architecture (Fig. 2, G and H), and still caused an overall increase in F-actin levels (Fig. 2I). R1055A/D1056A also showed no significant effect in cells (Fig. 2, A and B). These cell-based experiments demonstrated that the conserved “RRKR” residues in the basic region are most likely critical for DAD function.

The mechanism proposed to account for the ability of DAD to induce actin filaments in cells involves binding of the DAD protein to cellular mDia proteins, whereby EGFP-DAD displaces intermolecular or intramolecular DID-DAD interactions (20). If this model is correct, then DAD variants that are inactive in the cell-based assays are because of their failure to bind DAD. This appears to be true for certain conserved residue variants found in the leucine-rich core of DAD (20).

To examine the potential contribution to DAD binding by these specific DAD residues, it was necessary to be able to quantify the affinity of the DID-DAD interaction. Similar to recent work (12), fluorescence anisotropy was successfully used to monitor the binding of DID to a fluorescein-labeled peptide (residues 1036–1061 of mDia2). To confirm that the peptide could function like the full-length DAD in cells, microinjection of the DAD peptide into mouse fibroblasts proved to elicit the same activation of actin fiber formation (data not shown). Therefore, the fluorescein-labeled DAD peptide provided a valid approach to correlating the function of DAD in cells with its ability to bind to DID. The addition of purified GST-DID (mDia1, residues 129–369) or GST-DID (mDia2, residues 140–396) increased the anisotropy of the DAD peptide in a concentration-dependent manner. As shown in Fig. 3A and described under "Materials and Methods," the anisotropy data were fitted with a one-step binding model yielding a $K_D = 0.28 \mu M$ for the interaction of the DAD peptide to GST-DID (mDia1) and $K_D = 0.29 \mu M$ for GST-DID (mDia2).

Once the DID/wild-type DAD interaction was quantified, it was possible to probe the contribution of specific amino acid residues in DAD. Whereas the wild-type DAD peptide displayed significant interaction with GST-DID from both mDia1 and mDia2, the M1041A and R1057E/H1058E DAD peptides failed to bind to the GST-DID (mDia1) (data not shown).

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shown) or GST-DID (mDia2) (Fig. 3B). For the M1041A and R1057E/ R1058E DAD peptides, the affinity for GST-DID was so low that it was impossible to properly calculate a valid $K_D$ for the interaction (see Table 1 for list of $K_D$ values). Taken together, these data demonstrated that in addition to the previously characterized core region of DAD, the basic region contributes significantly to interactions with DID. The binding assays also provided a valid correlation between DID-DAD binding and the ability of DAD to induce actin filaments in cells.

The Positive Charges of the Basic Residues RRKR Are Important to DID Binding—Whereas it was observed that the double glutamate mutants of DAD, R1057E/R1058E and K1059E/R1060E, lacked the ability to bind to DID and affect the cellular actin architecture, it was prudent to further investigate the importance of each amino acid residue in the DAD basic region. In addition, if these RRKR residues are involved in DID-DAD binding, are the positive charges associated with these residues important to the interaction? To address this question, single alanine and single glutamate mutations were made on all four 1057RRKR1060 residues. As shown in Fig. 4, A and B, the binding affinity of DAD peptides corresponding to R1057A and all glutamate single substitutions were tested for in vitro binding to GST-DID (mDia2-(140–396)). All of the single mutant DAD peptides could be fitted with a single site binding model. Whereas the R1057A DAD peptide had a slightly decreased affinity, all of the single glutamate variants had even lower DID binding affinities, with the R1057E and R1058E DAD peptides exhibiting a 10- and 12-fold decrease in affinity, respectively (see Table 1 for a list of the $K_D$ values).
Because the glutamate substitutions exhibited lower binding affinity to DID, the correlation to the ability of these DAD variants to affect the cellular actin architecture was studied. A representative experiment is shown in Fig. 5, A–F. Whereas the R1057A DAD did show a small decrease in DID binding affinity, it still caused the accumulation of F-actin as shown in Fig. 5, C and D. As predicted by the binding studies, a single glutamate substitution blocked the ability of EGFP-DAD to effect F-actin (Fig. 5, E and F). Likewise, all of the single glutamate mutant EGFP-DAD constructs failed to induce actin accumulation as summarized in Fig. 5G. The glutamate mutations appeared to decrease the DID binding affinity to a critical threshold that resulted in the inability of DAD to induce changes in the cellular actin architecture. Whereas the single alanine mutations did not reduce the DID-DAD affinity enough to lose the cellular effects, it would appear that the change in charge from a Arg (or Lys)/Glu produced a more significant disruption in its interactions to DID.

To address if the removal of another positive charge in the basic region of DAD, generated without an associated change in charge, could cause a disruption in its cellular function, a double alanine mutant (R1057A/R1058A) was tested. In cells, this double alanine mutant showed no ability to induce cellular actin filaments (data not shown). This result indicates that whereas the change in charge (glutamate mutants) disrupts DID-DAD binding, the presence of a significant positively charged patch in the basic region is required for efficient interactions with DID.

**TABLE 1**

Dissociation constants for the binding of GST-DID (mDia2-(140–396)) to DAD peptide-(1036–1061), as determined by fluorescence anisotropy

| DAD mutation | $K_D$ (μM) |
|--------------|------------|
| Wild type    | 0.29       |
| M1041A       | >20$^a$    |
| R1057A       | 0.78       |
| R1057E       | 2.78       |
| R1058E       | 3.50       |
| K1059E       | 1.50       |
| R1060E       | 1.26       |
| R1057E/R1058E| >20$^a$    |

$^a$ The M1041A and R1057E/R1058E DAD mutants exhibited little to no binding to GST-DID, making it impossible to determine a reliable $K_D$ for these binding interactions.

FIGURE 4. Analysis of specific residues in the basic region in their contribution to the binding of DID. A, composition of the DAD peptide used in the DAD-DID binding experiments (fluorescence anisotropy). Specific amino acid residue mutations are indicated below the peptide. B, purified GST-DID from mDia2-(140–396) was added to 10 nM of the indicated DAD peptide in TNM buffer. As described under “Materials and Methods,” all anisotropy measurements were transformed into a fraction of DAD peptide bound and plotted versus the concentration of free GST-DID protein. The resulting $K_D$ values for each mutant DAD peptide-DID (mDia2) complex are listed in Table 1.

FIGURE 5. DAD basic region mutants that have decreased binding affinity to DID fail to affect F-actin assembly. A–F, F-actin assembly was observed following expression of EGFP-DAD fusion proteins, as described under “Materials and Methods.” EGFP expression (A, C, and E) is shown on the left; TRITC-phalloidin staining on the right (B, D, and F). The bar represents 10 μm. G, F-actin accumulation from cells expressing the indicated DAD variants. Versions of EGFP-DAD bearing amino acid substitutions that lowered the ability of DAD to bind to DID failed to stimulate an elevation in F-actin levels.
Expression of Full-length Versions of mDia2 with DAD Mutations That Block Binding to DID Triggers the Assembly of Filopodia-like Structures—To examine the effects of disrupting the ability of DAD to interact with DID in the context of full-length protein, the R1057A, R1057E/R1058E, and M1041A substitutions were introduced into mDia2 expression plasmids. Protein expression and F-actin architecture in cells 4 h after microinjection of the respective vectors into NIH 3T3 cells maintained in 0.1% fetal calf serum are shown Fig. 6, B and C. As expected, expression of mDia2-R1057A (Fig. 6B) had no effect on F-actin assembly in comparison to neighboring un.injected cells or cells expressing mDia2 (Fig. 6A). In contrast, cells expressing either mDia2-R1057E/R1058E or mDia2-M1041A caused the disruption of pre-existent stress fibers and the assembly of extensive filopodial-like structures at the cell periphery. To ensure that the structures were not because of retraction of the lamella, time lapse microscopy (Fig. 6, E and F; supplemental Movie S1) of cells expressing a version of YFP-mDia2-M1041A showed that the deregulated form localizes to the tips of structures that elongate away from the cell edge. These results are consistent with the idea that mDia2 is autoregulated via DAD interactions with DID. Moreover, the observed induction of filopodia assembly in the absence of other extracellular stimuli further supports the observation that mDia2 has an important role in the formation of microspikes and filopodia (30–32).

**DISCUSSION**

The current study provides additional evidence that DAD acts as a key component of the regulatory mechanism that controls actin remodeling by mDia family members (12, 20). In addition to the observation that conserved residues within the leucine-rich core of DID were critical for binding to amino-terminal regions of mDia1 and mDia2, we show here that the conserved basic region is also required for binding to DID and its ability to participate in autoregulation. Indeed, as we have shown here, expression of full-length mDia2 with DID binding defective versions of DAD causes mDia2 deregulation and an induction of filopodia assembly.

The observation that the DAD peptide derived from the mDia2 sequence could bind to mDia1-DID was not surprising. First, the sequences of the conserved region of DID from mDia1 and mDia2 are almost identical (see Fig. 1B). In addition, our previous study demonstrated that the microinjection of DID from mDia2 into mouse fibroblasts activates actin fiber formation through endogenous mDia1 (20). Therefore, although the GBD-DID regions of mDia1 and mDia2 may contain some degree of difference, the contribution of DID to the autoregulation process appears to be via the same DID-binding mechanism for both molecules.

Recent structural and biochemical studies of the NH2-terminal regions of mDia1 have clearly demonstrated that a dimerization domain exists adjacent to the GBD-DID regions (12, 21, 22). Whereas the DID constructs used in this study do not contain the dimerization domain, their DID binding affinities are similar to the reported Kd values of 0.25 (12) and 1.6 μM (21) for the binding of an mDia1-DAD peptide to a dimeric mDia1 DID-DD construct. In addition, it has been recently reported that both monomeric and dimeric mDia1 DID constructs bind to RhoA with similar affinities (22). Therefore, dimerization of the NH2 termini of mDia by the dimerization domain does not seem to facilitate a cooperative binding process with either Rho or DAD (22).

The characterization of the basic region should further elucidate the contribution of DAD to autoregulation. Alterations in this region that block binding to DID interfere with the ability of exogenously expressed EGFP-DAD fusion proteins to activate actin filament assembly. This result was consistent with the original characterization of DAD (20), where alanine replacement of the conserved methionine (M1041A) blocked its effects upon expression in cells. Together with observations that DAD can activate Bni1p-dependent actin cable assembly in budding yeast (27) and that DAD peptides can deregulate or activate autoinhibited mDia1 *in vitro*, these experiments show that the isolated DAD can unlock the mDia autoregulatory mechanism in *trans* (12). The observation provides a certain utility to understanding where and how DAD, acting as an “anchor” for autoinhibition as proposed by Li and Higgs (12), interacts with DID. In addition, it gives additional insight into how activated GTP-bound Rho family members bind to adjacent NH2-terminal (GBD) domains and induce the release of DAD-DID interactions, thereby alleviating the inhibitory effects on FH2-mediated actin nucleation and filament elongation. Based on the known structure of the mDia1 DID (21, 22), NMR cross-saturation and biochemical studies (22), and the predicted α-helical leucine-rich and basic regions of the DAD peptide, it is now possible to make some predictions as to the nature and location of the DAD-DID interactions.

In this study, by testing single alanine and glutamate mutations in the 1057RRKR1060 basic region of mDia2, it was observed that while simply changing a basic residue an alanine might slightly decrease the DID-DAD affinity, it was not enough to prevent the ability to induce effects in the cellular actin architecture. However, by individually changing the charge of basic residues of an acidic group, the decreased DID binding affinity effectively blocked the induction of actin filament formation. The same situation was true for the double alanine mutation, R1057A/R1058A. Clearly, the data have demonstrated that the basic amino acid residues in 1057RRKR1060 are important in the binding of DID, but the individual alanine versus glutamate mutations may also shed some light on the nature of the interactions. It would seem that if the basic region residues were primarily involved in hydrogen bond interactions with DID, then the alanine mutations would have probably decreased the DID-DAD affinity at least as much as a glutamate substitution. Because the glutamate mutations decreased the DID-DAD affinity and exhibited no induction of cellular actin filaments, the basic residues are likely involved in an ionic or electrophilic interaction(s) with an acidic group(s) in DID. The fact that a substitution of an acidic group for each basic residue would add a repulsory force agrees with the glutamate mutations having lower DID binding affinities. Whereas it is evident that the basic region is important for efficient DID binding, it has already been shown that nonpolar residues in DAD play a critical role in binding to DID. As a helical wheel analysis of DAD would predict that the important residues in the core region (Met1041 and Leu1044 (20)), might exist on a hydrophobic side of a helix, and that AlaL1057 and Leu1059 of mDia1 DID have been characterized as being essential for DID binding (21, 22), it seems likely that critical hydrophobic interactions exist at the DID-DAD interface. This study extends the likely DID-DAD interactions to include ionic interactions as well.

Whereas our studies have demonstrated that the four basic residues in mDia2 (1057RRKR1060) are involved in the binding of DID, our findings have not elucidated the exact manner in which these residues contribute to DID binding. As discussed earlier, the most probable explanation would involve the formation of electrostatic interactions between the basic amino acid residues in DAD and acidic residues on the surface of the DID domain. While the idea of an interaction of the basic DAD residues with a complementary negatively charged surface on the DID domain is very plausible, the specific residues in DID are unknown. Using the combined knowledge of the structures of mDia1 DID (21, 22) with the fact that the mDia1 DID mutations, A256D and...
I259D, significantly reduced the binding affinity for DAD (21, 22), it is only possible to speculate potential residues in DID that could interact with the basic region of DAD. Whereas there are many negatively charged residues on the surface of the DID domain, a small region of fairly conserved acidic residues (mDia2, Glu279, Glu280, and Glu284; mDia1, Glu264, Asp265, and Glu266) that exists on a turn near the proposed binding residues of Ala273-Ile276 (mDia1, Ala256-Ile259) might be able to interact with the basic region of DAD. However, as it has been shown that the E264K mutation in mDia1 (mDia2, Glu279) did not significantly affect DAD binding (21), the specific residues in DID that

FIGURE 6. Expression of full-length mDia2 DID-binding defective versions of DAD causes extensive remodeling of F-actin at the cell periphery. A–D, F-actin assembly was observed following expression of myc-tagged mDia2 or its variants with the indicated amino acid substitutions for 4 h following microinjection of each respective expression plasmid; cells were fixed and stained for expression with rabbit anti-Myc as previously described (25). The bars represent 10 μm. E and F, single frames from time lapse image acquisition of YFP-mDia2-M1041A expressed for 4 h as shown in supplemental Movie S1 (inset), which corresponds to the region shown in the inset (F).
might interact with the basic region of DAD will have to be identified by further DID mutation/binding studies or the structural determination of the DID-DAD interactions.

Although it is likely that the conserved basic region in DAD is directly involved in the binding of DID, it is also possible that the basic residues function to stabilize a conformation of the core residues in DAD that actually form the interaction with DID. If this were the case, there are potential acidic residues within DAD that are conserved (see Fig. 1B) and could form ionic interactions with the basic residues (1057RRKR1069). Conserved in the mDia proteins, mDia2 Asp1036 and Glu1037 could interact with the basic region. However, an even more likely couple of residues would be mDia2 Asp1042 and Glu1046. These residues are conserved throughout the mammalian mDia proteins, as well as the fruit fly diaphanous and yeast Bni1 and SepA proteins. The acidic residues, Asp1042 and Glu1046, also exist in a putative helical segment of DAD containing core residues important to DID binding (Met1041 and Leu1044) (Ref. 20 and this study). Interactions formed between these acidic residues and the basic region of DAD might serve to “lock” the appropriate helical conformation of the core residues of DAD to increase the affinity of DID binding. This would also be consistent with glutamate substitutions in the basic region of DAD having a more pronounced negative effect on the binding of DID.

Although many mutations in DID have been shown to decrease DID binding (22), we hypothesize that amino acid substitutions in these DAD-binding sites in a full-length mDia would also “activate” the formin similar to what we have observed here with mDia2-R1057E/R1058E and -M1041A. Likewise, we also expect that mutations in DAD that block the ability to bind to DID, in the context of full-length mDia2, would maintain mDia2 in the activated form regardless of the absence of GDP binding. Because DAD interferes with growth factor receptor trafficking, proliferation, and the motility of tumor cells, understanding the characteristics of DID-DAD interactions will further the development of DAD peptidomimetics as potential anti-cancer therapeutics.

In conclusion, we have uncovered additional molecular requirements for the diaphanous autoregulatory domain. Future studies using deregulated versions of other mDia proteins should allow further characterization of the roles of individual formins in cytoskeletal assembly. These studies also suggest alternative regulatory mechanisms where other cellular proteins or factors bind to or modify regions within (or adjacent to) DAD that alter its ability to interact with DID. In yeast, Bni1p interacts with Bud6 in a region near DAD and has the ability to activate the formin in vitro and in cells (6). It will be of interest to determine whether mammalian cells harbor similar biological activities.

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4 H. Holman and A. S. Alberts, unpublished observation.

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**Molecular Requirements for DAD-DID Binding**

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