mDia-interacting Protein Acts Downstream of Rho-mDia and Modifies Src Activation and Stress Fiber Formation*

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The formin homology protein mDia is a Rho GTPase effector protein that participates in stress fiber formation, cytokinesis, and transcriptional activation of the serum response factor. Although the function of another effector of Rho, Rho-associated kinase, is well established, relatively little is known about the functional mechanism and the downstream targets of mDia. Our recent report of a Rho-mDia-Src-tyrosine kinase pathway suggested an important role for mDia in cell adhesion turnover. We identified a new mDia-interacting protein which is expressed ubiquitously. The new protein mainly binds to the proline-rich region of mDia through its Src homology 3 domain and also binds to Grb2 through its proline-rich domain. The protein is localized at the cell periphery and membrane ruffles and co-localizes with mDia. Co-expression of vSrc and the mDia-interacting protein induces significant morphological changes at focal contacts and activation of vSrc. Furthermore, we found that the mDia-interacting protein plays an important role in stress fiber formation induced by active mDia1. Our results suggest that this new protein regulates actin polymerization and cell adhesion turnover in the downstream portion of the Rho-mDia pathway by interacting with Grb2 and Src.

The small GTPase Rho participates in cytoskeletal reorganization during cell adhesion, spreading, cell movements, and cytokinesis (for review, see Refs. 1, 2). The well known effects of Rho are stress fiber and focal adhesion formation. Many downstream effectors of Rho have been identified in the last several years, and they have been shown to mediate some of these Rho effects (for review, see Refs. 3, 4). In particular, the mechanism of stress fiber formation was elucidated to some extent by the identification of Rho-associated kinase (5, 6) and mDia (7). Rho-associated kinase causes the contraction of preexisting actin filaments, whereas mDia induces de novo actin polymerization (8, 9).

mDia is a mammalian homologue of Drosophila diaphanos.
RESULTS AND DISCUSSION

To identify novel mDia-binding proteins, we performed a yeast two-hybrid screen using a HeLa cell library with an active mDia2 fragment as bait (Fig. 1A, arrow). One positive clone (2Y1; Fig. 1A, arrow) arising from this screen appeared to be truncated at its 5’ end. This clone was therefore used as a hybridization probe to isolate a full-length clone (P3) from brain and placenta libraries. The P3 clone encoded a putative 722-amino acid protein that we named DIP. The 2Y1 and P3 clones differed at their C-terminal regions and appeared to represent splice variants of the same protein. The full-length protein has an N-terminal lyn-like Src homology 3 (SH3) domain (amino acids 1–58) followed by a bipartite nuclear localization signal (amino acids 171–192), a proline-rich domain (PRD; amino acids 170–249), and a leucine-rich domain (LRD; amino acids 505–717; Fig. 1A). While this work was in progress, Sano et al. (18) reported the identification of a leukemia-related protein called AF3p21 (GenBank™ accession number HSU 04737) with a sequence that is identical to our P3 clone. DIP mRNA (3.0 kilobases) was expressed in all tissues examined but was highly expressed in brain, heart, skeletal muscle, kidney, and liver (Fig. 1B). In addition, by probing immunoblots with two different DIP-specific antisera, we were able to identify a specifically immunoreactive protein of ~80 kDa in lysates from different cell lines (Fig. 1C). This broad expression pattern resembles that of mDia1 but is distinct from that of mDia2, the expression of which is largely confined to testis and cancer cell lines (10). When an active subregion of mDia1 (10) was used as bait in the yeast two-hybrid assay, the cells died, as previously reported for Bni1, a yeast homologue of mDia (19, 20). This may be because mDia1 has more proline-rich repeats than mDia2. Therefore, we performed subsequent experiments using mDia1 as the partner of DIP.

To confirm that DIP interacts with mDia, and to determine the nature of that interaction, we first used a GST pull-down assay. As shown in Fig. 2A, a, lane 2, C, the DIP SH3 domain alone bound strongly to the region of mDia that includes the FH1 domain (mDia1 region 2; Fig. 1A). The combination of the SH3 domain and PRD domain of DIP interacted weakly with the same FH1 domain-containing portion of mDia1 (lane 4, C), but this interaction was significant compared with other parts of mDia1 (lane 4, B and D). We observed a similarly weak but significant interaction between the LRD domain of DIP and the C-terminal portion of mDia (mDia region 3; Fig. 1A), which contains the FH2 domains (lane 5, D). To further confirm the interaction between DIP and mDia in mammalian cells, we transiently co-expressed HA-tagged DIP with Flag-tagged mDia1 in COS cells and assayed for complex formation by IP and IB (Fig. 2B). Flag-mDia1 was detected in full-length DIP (Fig. 2B, lane 3) and ΔLRD DIP (lane 5)-containing lysates immunoprecipitated with anti-HA Antibody (Ab). Because the PRD of DIP contained potential Grb2 binding sequences, we next tested whether DIP was physically associated with Grb2. A GST pull-down assay demonstrated a strong interaction between Grb2 and PRD of DIP (Fig. 2C, a). Furthermore, in vivo interaction between these two proteins was detected by IP (Fig. 2D). DIP but not ΔPRD, DIP was co-precipitated with Grb2 (Fig. 2D, lanes 2 and 3). We also found evidence for an intramolecular interaction between the SH3 region and PRD of DIP using both the yeast two-hybrid system and GST pull-down assays (data not shown). Accordingly, we constructed a schematic model for binding of DIP to mDia1 and Grb2 (Fig. 2E). A similar series of experiments suggested that mDia2 binds to DIP in the same manner (data not shown).

To investigate the subcellular localization pattern of DIP in mammalian cells, we transiently expressed GFP-DIP in HeLa cells and simultaneously detected endogenous mDia1 by indirect immunofluorescence with anti-mDia1 Ab. As shown in Fig. 3A, a, DIP was largely co-localized with mDia, especially at the cell periphery. Endogenous DIP was stained with anti-DIP-P Ab in late mitotic cells (Fig. 3A, b). Pronounced DIP immunoreactivity was observed at membrane ruffles.
We also examined whether DIP could compete with Src for binding to mDia. HeLa cells were transiently transfected with DIP and vSrc. Unexpectedly, DIP was consistently found to be co-localized with vSrc. Furthermore, co-expression of vSrc and DIP produced a unique morphology at the cell periphery compared with vSrc overexpression alone (Fig. 3b, a and b). We next biochemically examined the Src activation by DIP (Fig. 3b, c). DIP overexpression with vSrc induced the increased amount of active Src detected by clone 29 monoclonal Ab (Fig. 3c, lanes 3 and 4). These data suggest that DIP acts in concert with vSrc to influence cell adhesion and cell spreading.

To evaluate the role of DIP in the downstream portion of the Rho-mDia pathway, we investigated stress fiber formation induced by mDia. Serum-starved HeLa cells were microinjected with plasmids containing GFP-DIP and active mDia1 cDNAs (Fig. 4). Stress fiber formation was observed in the cells co-expressing active mDia1 and full-length DIP (Fig. 4, a and b). However, it was less pronounced in the cells co-expressing mDia1 and either ΔSH3-DIP or ΔPRD-DIP (Fig. 4, c and d) and was completely suppressed in cells co-expressing mDia1 and a mutant form of DIP lacking the SH3 to PRD domain (LRD-DIP; Fig. 4e). The attenuation of the stress fiber formation was restored by co-expression with ΔLDR-DIP (Fig. 4f). These data suggest that DIP, acting through its SH3 and PRD domains, is important for stress fiber formation in the downstream portion of the Rho signaling pathway.

In this study, we identified a new binding partner of mDia, DIP, and showed that this protein plays an important role in stress fiber formation. We further suggest that DIP is important for focal adhesion formation as part of a mDia-Src pathway on the basis of our observation that overexpression of vSrc with DIP induced morphological changes at focal contacts and activation of vSrc. Given the large body of data suggesting that Src is important for focal adhesion turnover (13–15), we think that a more detailed examination of Rho-mDia-Src pathway will contribute to the understanding of the mechanisms of focal adhesion turnover and cell migration.

A rat homologous protein of DIP (neural Wiskott-Aldrich syndrome protein (N-WASP)-interacting SH3 protein; 78% identity at the amino acid level) with an mRNA expression pattern that was restricted to brain and testis and was distinct from that of DIP, was recently identified by Fukuoka et al. (21). N-WASP-interacting SH3 protein was reported to induce microspike formation on epidermal growth factor-stimulated actin polymerization in a Cdc42-independent manner. N-WASP-induced actin polymerization mediated through the Arp2/3 complex is well established (22), and this event can occur independently from Cdc42 (23). Furthermore, N-WASP-interacting SH3 protein was proposed to be able to activate the Arp2/3 complex through an N-WASP-independent pathway (21). The relevance of N-WASP interaction to N-WASP-interacting SH3 protein function therefore remains to be established. Grb2 has
also been proposed to enhance Arp2/3 complex-mediated actin polymerization (24). Our data suggest that the N-terminal region of DIP is important for stress fiber formation. Although mDia is reported to participate in actin polymerization through the binding of its FH1 domain to profilin (7), the presence of profilin alone is not sufficient for the elongation of actin filaments to occur (22). Therefore, Grb2 and Arp2/3 may participate in rapid actin polymerization and stress fiber formation in the downstream portion of the Rho-mDia pathway by interacting with the DIP N terminus.

Finally, Grb2 is well recognized as an important signaling molecule, especially in adhesion signaling through Src (13–15) and in Ras signaling (25). Thus, we propose the existence of cross-talk between the Rho-mDia-DIP and Ras-PI3 kinase-Rac pathways.

**FIG. 3. Co-localization of DIP with mDia and activation of Src by DIP.** A, co-localization of DIP and mDia1. HeLa cells were transfected with GFP-DIP, and endogenous mDia1 (red) was immunostained with a specific anti-mDia1 Ab. b, mitotic HeLa cells were co-immunostained with anti-DIP-P (green) and anti-vinculin (red) Abs. In this merged image, yellow indicates co-localization. R, co-localization of DIP and Src. a, transfected vSrc and endogenous DIP. b, co-transfected vSrc and GFP-DIP. Endogenous DIP (green) was detected with anti-DIP-P Ab (a), and Src (red) was detected with anti-Src Ab (a and b). All photographs consist of merged images. C, COS7 cells were transfected with vSrc alone, DIP alone, or both vSrc and DIP as indicated. Src was immunoprecipitated with anti-Src polyclonal Ab (SRC-2) and immunoblotted with clone 327 monoclonal anti-Src Ab (active Src-IB) or clone 29 monoclonal anti-active Src Ab (active Src-IB).

**FIG. 4. Regulation of mDia1-induced stress fiber formation by DIP.** Active mDia1 was coexpressed with the indicated GFP-fused DIP proteins (b–f) or with GFP alone (a) in serum-starved HeLa cells by microinjection. a, active mDia1 with a GFP vector; b, with GFP-DIP; c, with GFP-ΔSH3 DIP (DIP lacking SH3); d, with GFP-ΔPRD DIP (DIP lacking PRD); e, with GFP-LRD DIP; f, with GFP-ΔLRD DIP (DIP lacking LRD). GFP signals were detected in the top panels. F-actin was stained with Texas Red-phalloidin in the bottom panels.
pathways (26) mediated by DIP-Grb2 binding. Future studies should aim to clarify the coordinated signaling events regulated by Rho family GTPases (Rho, Rac, and Cdc42) during cell adhesion and migration.

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