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Interaction of D-lactate dehydrogenase protein 2 (Dld2p) with F-actin: implication for an alternative function of Dld2p

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Abstract

D-Lactate dehydrogenase protein 2 [Yeast 15 (1999) 1377; Biochem. Biophys. Res. Commun. 295 (2002) 910] was initially identified as the actin interacting protein 2 (Aip2p) using a two-hybrid screen to search for proteins that interact with actin [Nat. Struct. Biol. 2 (1995) 28], but no other evidence indicating an interaction between Aip2p and actin cytoskeleton has been reported so far. During our search for the protein conformation modifying activity, we serendipitously identified Aip2p isolated from *Saccharomyces cerevisiae* as exhibiting an interaction with F-actin both in vitro and in vivo. Incubation with Aip2p facilitated the formation of the circular form of F-actin in vitro, which exhibited an aberrant trypsin susceptibility. Overexpression of Aip2p induced multi-buds in yeast cells, whereas reduced expression interfered with the formation of the cleavage furrow for the cell division, which was rescued by the introduction of wild-type Aip2p. While Aip2p-treated F-actin in the circular form was negligibly stained by rhodamine-labeled phalloidin (rhodamine–phalloidin) in vitro, rhodamine–phalloidin staining profiles in actin interacting protein 2 gene (AIP2)-modified cells suggested a correlation between the conformation of F-actin and the expression of Aip2p in vivo. AIP2-deleted cells became sensitive to osmotic conditions, a hallmark of actin dysfunction. Finally, immunoprecipitation of yeast cells using anti-Aip2p antibody demonstrated that Aip2p associates with actin. These properties suggest that Aip2p may interact with F-actin in vivo and play an important role in the yeast cell morphology.

Keywords: D-Lactate dehydrogenase protein 2; Actin interacting protein 2; Actin interacting protein 2 gene; F-actin; Trypsin susceptibility assay; Rhodamine–phalloidin staining; Yeast cell morphology

The actin cytoskeleton plays diverse roles in the cell, mediating endocytosis, exocytosis, cell motility, cell polarity, and cytokinesis, each in a spatially localized and temporally controlled manner [4]. Each of these events requires regulation of specific dynamic properties and spatial organization of actin filaments by members of a large collection of actin-binding proteins.

Actin interacting protein 1 (Aip1p) was originally identified using a two-hybrid screen, which has a distinct interaction footprint on actin subdomains III and IV, and is required for normal localization of cofilin to cortical actin patches as well as stimulation of cofilin activity [5]. Aip1p has also been proposed to control actin depolymerization in vivo, and plays an important regulatory role in the rapid remodeling of the cortical actin meshwork [6]. The crystal structure of Aip1p from *Saccharomyces cerevisiae* (*S. cerevisiae*) reveals that overall folding is mediated by two connected

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seven-bladed propellers [7]. The gene (YDL178w) encoding actin interacting protein 2 (Aip2p) was also identified using a two-hybrid screen to search for *S. cerevisiae* proteins that interact with actin [3], but there has been no direct evidence indicating an in vivo interaction between Aip2p and actin cytoskeleton so far. Subsequently, the YDL178w gene product has been reported to localize on mitochondria and exhibit D-lactate dehydrogenase (DLD) activity in vitro, and therefore renamed as D-lactate dehydrogenase protein 2 (Dld2p) [1]. The AIP3 gene encodes actin interacting protein 3 (Aip3p), which is identical to BUD6 that functions in bipolar bud selection in yeast diploid cells [8–10]. Aip3p is not essential for mitotic growth but is necessary for normal morphogenesis, and plays an important role in actin-directed polarized cell growth in yeast cells.

We previously developed an in vitro protein conformation modifying assay that measures the factor-dependent increase in protease susceptibility of a substrate as a criterion for activity [11–13]. Serendipitously, we isolated a chaperone that alters the conformation of protein substrates such as F-actin in vitro. This purified chaperone is the Aip2p [1,3]. The activity of the isolated Aip2p could alter the conformation of F-actin in vitro and the rhodamine–phalloidin staining profiles as well as its osmotic sensitivity in vivo, which seems to be involved in yeast cell morphology.

Materials and methods

Yeast strains and antibodies. Wild-type yeast strains (ATCC24657 for the wild-type strain, ATCC96099 and ATCC 96100 were mated for the diploid cells) used in this study were purchased from American Type Culture Collection. Protease deficient strain SH2777 was a gift from Dr. Harashima, Osaka University. Anti-actin antibody was purchased from Chemicon. Anti-Aip2p antibody was raised against the synthetic peptide corresponding to the C-terminal 15 amino acid residues of Aip2p (VHYDPNGILNPYKYI) which were coupled through a COOH-terminal cysteine residue to BSA.

Purification of hexahistidine-tagged Aip2p. In an effort to obtain sufficient quantities of Aip2p, the protein was prepared from the expression strain in yeast under control of the ADH promoter. The C-terminally hexahistidine-tagged YDL178w gene was amplified by PCR and inserted into the aureobasidin A (Ab A) selective expression vector pAUR123 (TaKaRa Biomedicals). The protease deficient strain SH2777 was transformed by this plasmid and transformants were grown on YPD plates containing 0.5 μg ml^{-1} Ab A. Inoculated medium (8 L) was incubated overnight at 30 °C to an OD at 600 nm of 1–2. Cells were collected, resuspended in 4 volumes of buffer B (50 mM NaPi (pH 8.0), 150 mM NaCl, and 10 mM imidazole), crushed using glass beads, and centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were collected and ultracentrifuged at 100,000*g* at 4 °C for 1 h. The precipitate was resuspended, passed through a Ni–NTA agarose column (Qiagen, K.K.) equilibrated in buffer B, and subsequently eluted with buffer B containing 0.5 M imidazole. Eluted fractions were dialyzed against buffer C (10 mM Hepes–KOH (pH 7.4), 50 mM NaCl, and 1 mM DTT), applied to an ion exchange Mono Q column (Amersham–Pharmacia Biotech, AKTA system) equilibrated with buffer C, and eluted with a linear NaCl gradient (100–500 mM). Immunoreactive fractions were dialyzed against buffer D (50 mM NaPi (pH 7.5), 10 mM NaCl, and 1 mM Mg $(OAc)_2$), and finally passed through a Superdex 200 gel filtration column equilibrated with buffer D.

Fluorescent microscopy. Rhodamine-labeled phalloidin (rhodamine–phalloidin) staining was performed as previously [14]. Samples were imaged with a Delta-Vision microscopy system (Applied Precision), with out of focus light pertaining to visualized images being removed by interactive deconvolution.

Formation of circular F-actin in vitro. Alexa 488-labeled G-actin (Molecular Probes) was converted into its Mg^{2+} form and polymerized for 2 h. Purified histidine-tagged Aip2p was added to Alexa 488-conjugated F-actin in the buffer E (10 mM Tris–Cl (pH 8.0), 0.1 M KCl, and 10 mM $MgCl_2$) and then incubated at 30 °C for 30 min in the presence of 1 mM ATP. Twenty microliter samples were then placed onto a glass-bottomed dish and covered with Slow fade anti-fade (Molecular Probes). To observe a staining profile of the circular F-actin with anti-Aip2p antibody, Alexa 594-conjugated anti-Aip2p antibody (red) was incubated with Alexa 488-conjugated F-actin (green). Affinity-purified Alexa 594-conjugated anti-Aip2p antibody was used at 1:100.

Trypsin susceptibility assay. Assays (200 μl) were initiated by adding 200 ng of polymerized rabbit muscle actin to buffer E containing 1 mM ATP and 500 ng of hexahistidine-tagged Aip2p, and incubated at 30 °C for 15 min. After incubation, samples were treated with trypsin (0.2 μg ml^{-1}) at 16 °C for 15 min. The reaction was terminated by incubation with soybean trypsin inhibitor (0.4 μg ml^{-1}) on ice for 5 min, TCA-precipitated with tRNA carrier, and then subjected to SDS–PAGE and Western blotting. To detect actin, affinity-purified polyclonal rabbit anti-actin antibody served as the primary antibody and horseradish peroxidase-linked IgG (ICN Pharmaceuticals) was the secondary antibody. Immunoreactive bands were visualized by ECL-plus (Amersham Biosciences) and analyzed using a Fluoro Smax (Bio-Rad).

Overexpression and disruption of actin interacting protein 2 gene in yeast cells. In an effort to obtain an Aip2p overexpressed strain, a cloned actin interacting protein 2 gene (AIP2) encoding Aip2p was inserted into the galactose inducible yeast expression vector pYES2. Yeast cells (ATCC96099) were transformed by this plasmid, selected on minimal plates without uracil, and checked by colony PCR. Aip2p was induced with 2% galactose at 30 °C for 6 h. To generate a strain containing a disrupted AIP2, the middle region of Aip2p (1039 base pairs) was substituted with a histidine marker gene fragment, and then inserted into pBluescript KS+ vector (Stratagene). A diploid strain was transformed with the linearized vector and the tetrads produced were then analyzed by Southern blotting to verify the presence of the disrupted gene.

Immunoprecipitation of Aip2p–actin complex. Yeast spheroplasts from wild-type or AIP2-deleted cells were gently homogenized in the buffer (10 mM Tris–Cl, pH 8.0, 0.1 M KCl, and 10 mM $MgCl_2$) followed by an centrifugation at 100,000*g* for 60 min at 4 °C. An antibody against the C-terminal peptide of Aip2p or an antibody against yeast actin (Chemicon) was conjugated to Formyl-cellulofine (Seikagaku-Kogyo) according to the manufacturer's instructions. The resin was equilibrated with immunoprecipitation buffer (20 mM Tris–HCl, pH 7.9, 75 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, and 8% sucrose) and then used for the immunoprecipitation.

Results

Aip2p–F-actin interaction in vitro

In the absence of Aip2p, polymerized F-actin displayed a linear structure (Fig. 1A, (−) Aip2p), whereas in the presence of Aip2p, de novo formation of circular F-actin was observed (Fig. 1A, (+) Aip2p). The average

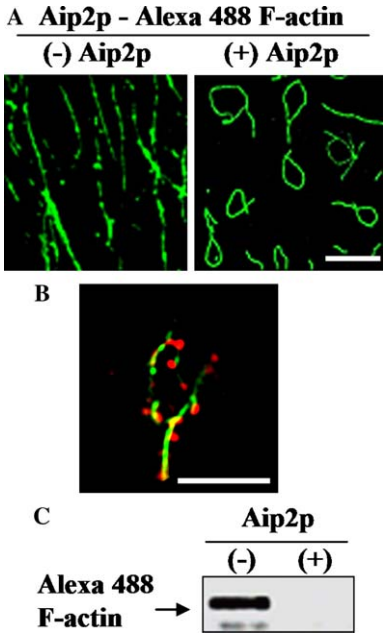

Fig. 1. (A) Formation of circular F-actin in vitro. Alexa 488-conjugated actin (Molecular Probes) was polymerized in high salt buffer (10 mM Tris–Cl (pH 8.0), 100 mM KCl, and 2 mM $MgCl_2$) and incubated with or without Aip2p in the presence of 1 mM ATP. Scale bar is 5 μm. (B) Anti-Aip2p antibody stains the circular F-actin in a discontinuous pattern. Alexa 594-conjugated anti-Aip2p antibody (red) was incubated with Alexa 488-conjugated F-actin (green) as described in Materials and methods. Samples were examined by fluorescent microscopy. Scale bar is 5 μm. (C) Trypsin susceptibility of Aip2p-bound circular F-actin is increased. Alexa 488-conjugated F-actin was incubated with Aip2p and ATP as described in Materials and methods. Samples were examined by fluorescent microscopy and subjected to the trypsin susceptibility assay.

radius of the circular F-actin was approximately 2–4 μm, which remained constant under the reaction conditions used in this study (Aip2p:F-actin = 1:1–1:5) (data not shown). The circularization process was very efficient as over 60% of the F-actin changed into circular form after incubation with Aip2p at 30 °C for 30 min. Alexa 488-conjugated anti-Aip2p antibody stained the circular F-actin in a discontinuous pattern (Fig. 1B). Incubation of Alexa 488-conjugated F-actin with Aip2p did not increase the rate of G-actin (see Fig. 1A). Alexa 488-conjugated F-actin was incubated with or without Aip2p as described above, treated with trypsin, and then subjected to Western blot analysis. The protease susceptibility of the Aip2p-bound "circular form" of F-actin clearly increased (Fig. 1C), indicating that the Aip2p-bound "circular form" of F-actin possesses an aberrant conformation compared to the linear form of F-actin.

Altered yeast cell morphology after the AIP2 modification

The phenotypic abnormalities were observed following modification of AIP2 expression. When Aip2p was overexpressed in yeast cells using the multicopy expression vector pYES2 and cultured under Aip2p-inducible conditions, cells produced multi-buds from the same mother neck or exhibited elongated bud necks (Fig. 2A, Overexpressed). This suggested that an excessive amount of Aip2p resulted in an abnormal budding process in yeast cells. In contrast, AIP2-deleted cells displayed deformed morphology reflecting an inability to properly form the cleavage furrow during cell division (Fig. 2A, Disrupted). The expression of intact Aip2p in the AIP2-deleted cells rescued this morphological defect (Fig. 2A, Plasmid rescue (AIP2)).

Because an abnormal actin cytoskeleton is considered as a major cause of such altered yeast cell morphology [15], the distribution of F-actin was examined in wild-type yeast cells, AIP2-deleted cells, and AIP2-overexpressed cells using rhodamine–phalloidin staining, which specifically stains F-actin in yeast cells. F-actin

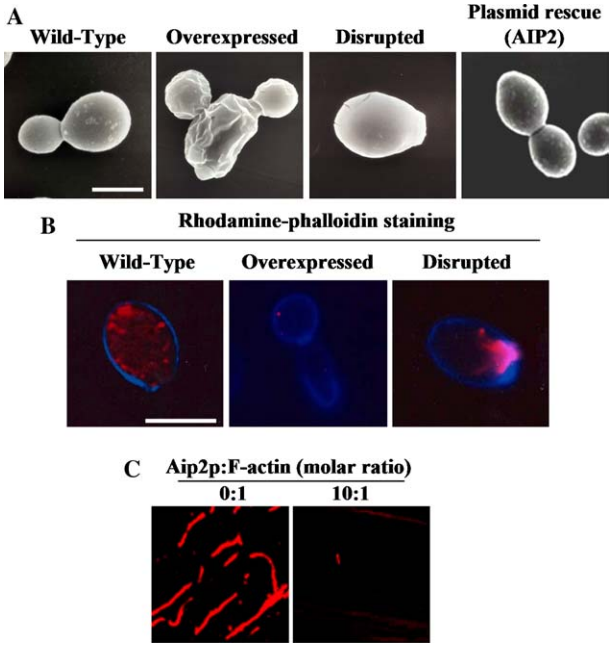

Fig. 2. (A) Wild-type (Wild-Type), AIP2-overexpressed (Overexpressed), and AIP2-deleted (Disrupted) strains were visualized using scanning electron microscopy (SEM). AIP2-overexpressed yeast cells produced multi-buds, while AIP2-deleted cells could not generate a proper cleavage furrow. The expression of AIP2 restored the cell morphology defect of the deleted strain (Plasmid rescue, AIP2). The AIP2-deleted strain was transformed with the pAUR123 plasmid carrying the gene for Aip2p under control of the ADH promoter. All transformants were cultured to an OD of 0.5–0.8 at 600 nm and then observed with SEM. Scale bar is 2 μm. (B) F-actin distribution with or without Aip2p in vivo. Wild-type yeast (Wild-Type), AIP2-deleted cells (Disrupted), and AIP2-overexpressed cells (Overexpressed) were incubated with 6.6 μM of rhodamine–phalloidin (red). Yeast cell wall was counterstained by Calcofluor White (Sigma) shown as blue. (C) F-actin distribution with or without Aip2p in vitro. Rabbit muscle actin (Molecular Probes) was polymerized, incubated with (10:1) or without (0:1) Aip2p in the presence of 1 mM ATP, and then stained with 6.6 μM of rhodamine–phalloidin.

was found to be clustered in the AIP2-deleted cells (Fig. 2B, right panel, Disrupted) compared to wild-type cells (Fig. 2B, left panel, Wild-Type). In contrast, when Aip2p was overexpressed in the wild-type strain using a multicopy expression vector, rhodamine–phalloidin-stained F-actin was only sparsely observed (Fig. 2B, middle panel, Overexpressed).

The in vitro rhodamine–phalloidin staining profile of F-actin on a glass slide varied considerably from that of F-actin incubated with Aip2p. Rhodamine–phalloidin hardly detected F-actin following incubation with Aip2p (Fig. 2C, right panel, 10:1) compared to the rhodamine–phalloidin staining profile of F-actin in the absence of Aip2p (Fig. 2C, left panel, 0:1). When fluorescently labeled G-actin was polymerized and used as a substrate (Alexa 488-conjugated F-actin), on the other hand, the fluorescent signals were not altered even after the incubation with Aip2p (see Fig. 1A). The total F-/G-actin contents remained at the same level after the AIP2-modification (data not shown).

Taken together, the incubation with Aip2p either competitively interferes with the rhodamine–phalloidin staining of F-actin or modifies the conformation of F-actin which the rhodamine–phalloidin hardly stained. The fact that the in vitro formation of circular F-actin (Fig. 1A) with its modified trypsin susceptibility (Fig. 1C) after the incubation with Aip2p supports the latter notion. Furthermore, the AIP2-deleted cells became sensitive to osmotic conditions (Fig. 3A), which is a hallmark of actin dysfunction [15]. Finally, immunoprecipitation of yeast cells using anti-Aip2p antibody demonstrated that Aip2p associates with actin (Fig. 3B, left panel). The reciprocal immunoprecipitation experiment using anti-actin antibody showed that Aip2p is contained in a complex with anti-actin antibody (Fig. 3B, right panel).

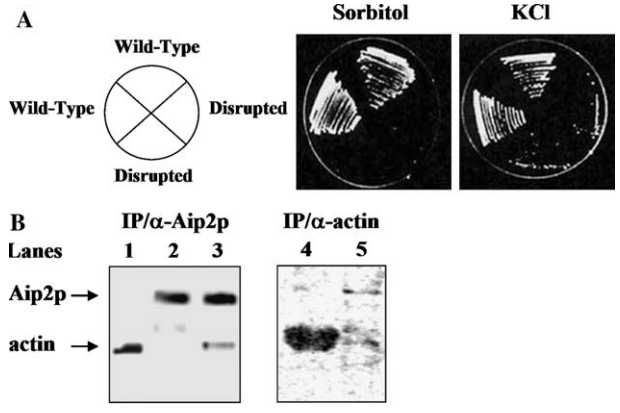

Fig. 3. (A) AIP2-deleted cells are osmotic sensitive. Wild-type (Wild-Type) and AIP2-deleted cells (Disrupted) were grown on rich media containing 1.8 M sorbitol or 1.2 M KCl at 30 °C. (B) Actin and Aip2p were detected by anti-actin antibody (α-actin, lanes 1, 3, and 4) and anti-Aip2p antibody (α-Aip2p, lanes 2, 3, and 5), respectively, in the complex immunoprecipitated with anti-Aip2p antibody (IP/α-Aip2p, lane 3), and in the complex immunoprecipitated with anti-actin antibody (IP/α-actin, lanes 4 and 5). Lanes 1 (actin) and 2 (Aip2p) are marker lanes.

Discussion

A two-hybrid screen originally identified the AIP2 that interacts with actin in *S. cerevisiae* proteins, and therefore it was classified as a member of actin interacting proteins [3]. Chelstowska et al. [1] reported that cell extracts expressing Aip2p exhibited DLD activity in vitro and renamed as D-lactate dehydrogenase protein 2 (Dld2p), even though its expression was not dependent on the Rtg proteins, which are known to be required for the expression of the CIT2 gene that encodes the peroxysomal isoform of citrate synthase. Flick and Konieczny [2] also found Aip2p, implicated with potential MLP/CRP3 interacting proteins, from a mouse cDNA library and exhibited DLD activity in vitro. It is well known that DLD activity is found in mitochondrial fractions. Nevertheless, the interaction between actin cytoskeleton and Aip2p has not been characterized by these efforts.

Given that Aip2p was initially identified as a member of actin interacting protein, several lines of evidence detailing the interaction between Aip2p and actin cytoskeleton were obtained and consisted of: (1) Aip2p-bound F-actin adopted a circular form with a resultant increase in trypsin susceptibility in vitro; (2) rhodamine–phalloidin-stained F-actin congregated in AIP2-deleted cells, however, when Aip2p was overexpressed using a multicopy expression vector, rhodamine–phalloidin-stained F-actin was only sparsely distributed; and (3) AIP2-deleted cells became osmotically sensitive. Schwikowski et al. [16] suggested that the function of Aip2p may be associated with cell polarity by a computational analysis of protein–protein interactions in yeast, which seems to be consistent with our results. It is possible that Dld2p/Aip2p may be multifunctional.

The actin cytoskeleton is relatively flexible compared to microtubules. With regard to in vitro actin manipulation, Ishiwata and colleagues [17] reported a laborious approach that established the formation of a super helix/circular form of F-actin in their motility assay system in which polymerized F-actin was bound to a silicone- or nitrocellulose-coated surface on which a torque component using heavy meromyosin and ATP was assembled to slide F-actin. Even in their well-defined system, formation of the super helix/circular form was determined by chance based on the balance between the magnitude of net torque and the rigidity of the actin filament. Consequently, circular F-actin is not easily produced. In contrast, we achieved the formation of circular F-actin simply by incubating the linear

conformer with Aip2p in the presence of ATP in vitro, a process that was 60–70% efficient over 30 min.

In this study, we have demonstrated that the Aip2p interacts with actin cytoskeleton. Moreover, our results provide the first evidence that Aip2p activity is involved in yeast cell morphology. Disruption or overexpression of the AIP2 significantly affected bud formation in yeast cells, which indicates that Aip2p is required for proper yeast cell morphology. The properties we have identified suggest that a more complete understanding of the activities of Aip2p may facilitate the analysis of the mechanisms that regulate F-actin dynamics in vivo.

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