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Oligomeric Aip2p/Dld2p modifies the protein conformation of both properly folded and misfolded substrates in vitro

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Abstract

Oligomeric actin-interacting protein 2 (Aip2p) [Nat. Struct. Biol. 2 (1995) 28]/D-lactate dehydrogenase protein 2 (Dld2p) [Yeast 15 (1999) 1377, Biochem. Biophys. Res. Commun. 295 (2002) 910] exhibits the unique grapple-like structure with an ATP-dependent opening [Biochem. Biophys. Res. Commun. 320 (2004) 1271], which is required for the F-actin conformation modifying activity in vitro and in vivo [Biochem. Biophys. Res. Commun. 319 (2004) 78]. To further investigate the molecular nature of oligomeric Aip2p/Dld2p, the substrate specificity of its binding and protein conformation modifying activity was examined. In the presence of 1 mM ATP or AMP-PNP, oligomeric Aip2p/Dld2p bound to all substrates so far examined, and modified the conformation of actin, DNase I, the mature form of invertase, prepro-α-factor, pro-α-factor, and mitochondrial superoxide dismutase, as determined by the trypsin susceptibility assay. Of note, the activity could modify even the conformation of pathogenic highly aggregated polypeptides, such as recombinant prion protein in β-sheet form, α-synuclein, and amyloid β (1–42) in the presence of ATP. The in vivo protein conformation modifying activity, however, depends on the growth stage; the most significant substrate modification activity was observed in yeast cells at the log phase, suggesting the presence of a cofactor/s in yeast cells, where F-actin is supposed to be a major target in vivo. These data further support our previous notion that the oligomeric Aip2p/Dld2p may belong to an unusual class of molecular chaperones [Biochem. Biophys. Res. Commun. 320 (2004) 1271], which can target both properly folded and misfolded proteins in an ATP-dependent manner in vitro.

Keywords: Actin interacting protein 2; D-Lactate dehydrogenase protein 2; Oligomeric form; Trypsin susceptibility assay; ATP-dependent conformation modifying activity; Broad substrate specificity; β-Sheet conformation; Cell-cycle dependent

The actin and its interacting proteins play diverse roles in the cell, mediating endocytosis, exocytosis, cell motility, cell polarity, and cytokinesis [6]. Among them, a search for *Saccharomyces cerevisiae* proteins that interact with actin in the two-hybrid system identified the gene encoding Aip2p (YDL178w) [1], and the same gene product has been reported to exhibit D-lactate dehydrogenase (DLD) activity in vitro in yeast cells [2] as well as in mammalian cells [3].

We previously reported that the Aip2p [1]/Dld2p [2,3] exhibits an interaction with F-actin both in vitro and in vivo with its unique grapple-like structure and an ATP-dependent opening [5]. Incubation with Aip2p/Dld2p

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facilitated the formation of the circular form of F-actin in vitro, which possessed an aberrant conformation compared to the linear form of F-actin. 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. Furthermore, the AIP2-deleted cells became sensitive to an osmotic condition, which is a hallmark of actin dysfunction, and Aip2p/Dld2p co-immunoprecipitated with actin in yeast cells.

Following ultrastructural analysis revealed a novel oligomeric grapple-like structure of 10–12 subunits with an ATP-dependent opening [4]. ATP regulates the opening and closing of the "gate" that forms the opening within oligomeric Aip2p/Dld2p, where binding to the substrate occurs while in the open form. In the presence of ATP (open state), oligomeric Aip2p/Dld2p bound the F-actin fiber within the opening, whereas in the absence of ATP (closed state), no binding was observed. Simultaneously, the oligomeric Aip2p/Dld2p increased the trypsin susceptibility of F-actin in an ATP-dependent manner that ATP-binding rather than ATP hydrolysis is required for the protein conformation modifying reaction.

During our consecutive investigation, it was suggested that the oligomeric Aip2p/Dld2p also bound and modified the conformation of several protein substrates other than F-actin as determined by the trypsin susceptibility assay in vitro. Here, we further examined the substrate specificity of its binding property and protein conformation modifying activity. Of note, the oligomeric Aip2p/Dld2p could target both properly folded and even pathogenic highly aggregated proteins and thus, it exhibited no obvious substrate specificity for its binding and robust protein conformation modifying activity in vitro.

Materials and methods

Yeast strain and antibodies. Protease deficient strain SH2777 was a gift from Dr. Harashima, Osaka University. Affinity-purified polyclonal rabbit anti-actin, anti-α-synuclein, and anti-heavy meromyosin (HMM) antibodies were purchased from Chemicon. Anti-cytochrome *c* antibody was purchased from BD Biosciences. Anti-SOD and anti-DNase I antibodies were purchased from Sigma Chemical. Affinity-purified polyclonal rabbit anti-invertase and anti-α-factor antibodies were raised against polypeptides harbouring 15 amino acid residues at the C-terminus, respectively. Anti-recombinant prion protein (PrP) antibody, K1, was rabbit polyclonal antibody raised against PrP residues 26–40 [7,8].

Preparation of substrate proteins. Amyloid β (1–28), amyloid β (1–42), cytochrome *c*, DNase I, malate dehydrogenase (MDH), and mitochondrial SOD were purchased from Sigma Chemical. α-Synuclein was purchased from Chemicon. HMM, luciferase, and the mature form of invertase were purchased from Wako Chemicals. Rabbit muscle actin was purchased from Molecular Probes. Two micromolar of rabbit muscle G-actin was polymerized in high salt buffer (10 mM Tris–Cl, pH 8.0, 100 mM KCl, and 2 mM $MgCl_2$) at 37 °C for 2 h, and used as F-actin. The gene fragments of hexahistidine-tagged ppαF and proαF were amplified by PCR, respectively, inserted into pET11a plasmid, expressed in *Escherichia coli* BL21(DE3) using the pET system, and purified according to the manufacturer's protocol (Qiagen, K.K.). Purified ppαF was dialyzed against buffer A (10 mM Hepes–KOH, pH 7.4, 1 mM DTT, and 1 mM $Mg(OAc)_2$) and subsequently used in the trypsin susceptibility protein conformation modifying assay. Recombinant PrP (rPrP) was purchased from Prionics, AG. The PrP solubilized in PBS was kept at 4 °C until circular dichroism detected over 50% of β-sheet contents in the rPrP, and then used as "PrP in β-sheet form."

Surface plasmon resonance. BIAcore 3000 system was used to analyze molecular interactions by means of surface plasmon resonance (SPR). Purified oligomeric form of Aip2p/Dld2p was covalently linked to a Sensor Chip CM5 (carboxymethylated dextran surface), with the use of amine coupling chemistry according to manufacturer's instructions. Samples for analyte proteins were diluted (10 μg ml^{-1}) in the running buffer (10 mM Hepes–KOH, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM EDTA, 0.005% surfactant P20, and 1 mM ATP), and injected over the surface at 4 °C with a flow rate of 5 μl min^{-1}. Each sensorgram was subtracted for the response observed in the control flow cell containing a blank surface and results were analyzed by using BIA evaluation SPR kinetic software (Biacore).

Purification of hexahistidine-tagged Aip2p/Dld2p and trypsin susceptibility assay. In an effort to obtain sufficient quantities of oligomeric Aip2p/Dld2p, the protein was prepared from the expression strain in yeast under control of the ADH promoter as previously described [4,5]. The trypsin susceptibility assay was performed as previously described [4,5,9–11]. Briefly, assays (200 μl) were initiated by adding 200 ng (if not indicated) of protein substrates to buffer B (10 mM Tris–Cl, pH 8.0, 0.1 M KCl, and 10 mM $MgCl_2$) containing 1 mM ATP and 500 ng of hexahistidine-tagged oligomeric Aip2p/Dld2p, 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 with corresponding antibody at 1:1000 (unless otherwise noted). Immunoreactive bands were visualized by ECL-plus (Amersham Biosciences) and analyzed using a Fluoro SMAX (Bio-Rad).

Results

Substrate recognition and binding of oligomeric Aip2p/Dld2p

To examine the in vitro oligomeric Aip2p/Dld2p-substrate-binding specificity in detail, we performed a surface plasmon resonance (SPR) assay with a wide variety of proteins as binding substrates including amyloid β (1–28), amyloid β (1–42), α-synuclein, cytochrome *c*, F-actin, G-actin, HMM, Invertase, MDH, ppαF, proαF, PrP, and SOD. In the presence of 1 mM ATP (see Fig. 1A) or AMP-PNP (data not shown), oligomeric Aip2p/Dld2p bound to all substrates so far exam-

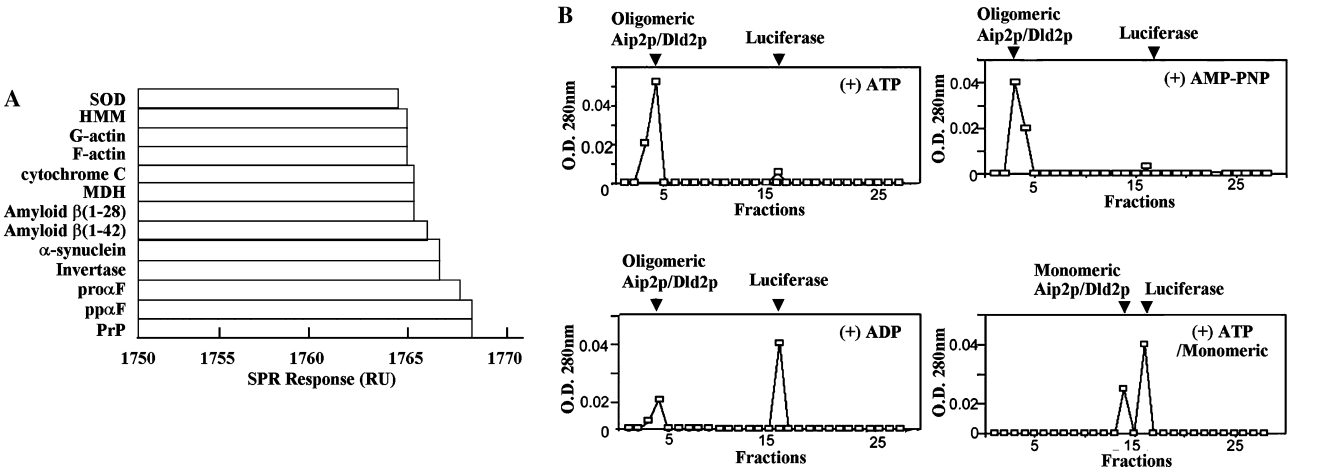

Fig. 1. Oligomeric Aip2p/Dld2p binds to all substrates examined in an ATP-dependent manner. (A) Interactions between oligomeric Aip2p/Dld2p and substrate proteins were measured by surface plasmon resonance (SPR) assay in the presence of ATP (see Materials and methods). SOD, superoxide dismutase; HMM, heavy meromyosin; MDH, malate dehydrogenase; proαF, pro-α-factor; ppαF, prepro-α-factor; and PrP, recombinant prion protein. (B) ATP requirement for its substrate-binding activity. Luciferase (300 ng) and oligomeric Aip2p/Dld2p (100 ng) were incubated in buffer B (see Materials and methods) with 1 mM ATP ((+) ATP), 1 mM AMP-PNP ((+) AMP-PNP), or 1 mM ADP ((+) ADP). Truncated monomeric Aip2p/Dld2p [4] could not bind substrate even in the presence of ATP ((+) ATP/monomeric). Each sample was passed through a gel filtration column (Superdex 200 column, Amersham BioSciences, Smart System).

ined, whereas oligomeric Aip2p/Dld2p bound to no substrates in the presence of ADP or with no nucleotides (data not shown). These data indicated that substrate-binding activity of oligomeric Aip2p/Dld2p requires ATP.

Gel filtration chromatography was then used to examine the oligomeric Aip2p/Dld2p–substrate interaction. Following incubation of luciferase with oligomeric Aip2p/Dld2p in the absence of ATP or presence of ADP, no oligomeric Aip2p/Dld2p–luciferase complex formation was observed, with both proteins being eluted separately (Fig. 1B, (+) ADP). In contrast, following incubation of both proteins in the presence of ATP or AMP-PNP, luciferase and oligomeric Aip2p/Dld2p eluted in fractions three and four as a complex (Fig. 1B, (+) ATP, and (+) AMP-PNP). Therefore, ATP and AMP-PNP induced the binding of oligomeric Aip2p/Dld2p to luciferase. This trend was also observed when ppαF and HMM were used as substrates (data not shown), indicating that substrate recognition is dependent on the binding of ATP to oligomeric Aip2p/Dld2p but not on ATP hydrolysis. In accordance with the result that the monomeric Aip2p/Dld2p with a C-terminal coiled-coil region-truncation failed to exhibit the conformation modifying activity [4], the coiled-coil region-truncated monomeric Aip2p/Dld2p exhibited no interaction with luciferase ((+) ATP/monomeric).

Oligomeric Aip2p/Dld2p has no obvious substrate specificity for its robust ATP-dependent protein conformation modifying activity in vitro

Oligomeric Aip2p/Dld2p increased the trypsin susceptibility of the substrate other than F-actin (ppαF, Fig. 2A, lanes 1, 2, and 4) in the presence of ATP. In the absence of ATP, however, the substrate was protected from trypsin digestion just as in the control (lanes 1 and 3), suggesting that this protein conformation modifying activity is ATP-dependent. Interestingly, further investigation revealed that the oligomeric Aip2p/Dld2p modified the conformation of actin, DNase I, the mature form of invertase, proαF, and mitochondrial SOD, as determined by the trypsin susceptibility assay (Fig. 2B). Thus, no obvious specific substrates have been identified for the protein conformation modifying activity of oligomeric Aip2p/Dld2p in vitro. Oligomeric Aip2p/Dld2p itself does not possess protease activity (Fig. 2C).

Use of the non-hydrolyzable ATP analog AMP-PNP yielded similar results to those observed with ATP, whereas the use of ADP failed to alter the luciferase activity (Fig. 2D), which is frequently used to analyze chaperone-mediated unfolding reactions as a properly folded protein substrate [12]. In accordance with our previous observation on the F-actin conformation modifying activity [4], these data further confirmed that ATP-binding rather than ATP hydrolysis is required for the protein conformation modifying reaction with other protein substrates, too.

In addition to the aforementioned indirect biochemical analyses suggesting that oligomeric Aip2p/Dld2p possesses protein conformation modifying activity, more direct evidence of this activity was sought through the use of low angle rotary shadowing [13]. Rabbit skeletal muscle HMM has a characteristic structure consisting of two globular heads and one tail (Fig. 3A, HMM). Following incubation with oligomeric Aip2p/Dld2p in the presence of 1 mM ATP, HMM heads were "unfold-

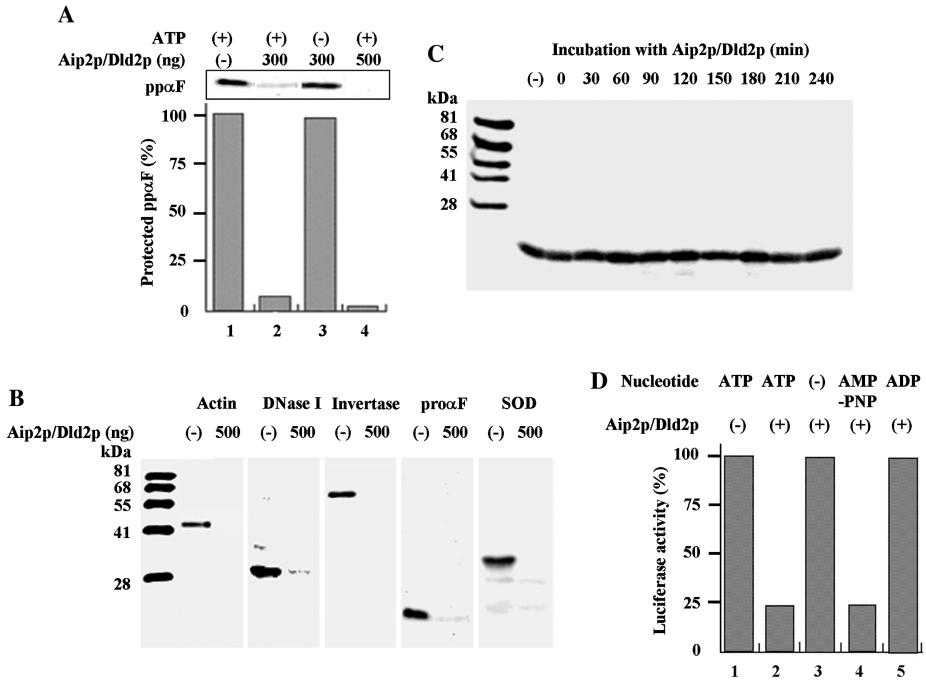

Fig. 2. Oligomeric Aip2p/Dld2p modifies the conformation of properly folded substrates in an ATP-dependent manner in vitro. (A) In the presence of ATP, Aip2p/Dld2p increases the protease susceptibility of ppαF (lanes 2 and 4). In the absence of ATP, no activity is observed (lane 3) and ppαF is resistant to trypsin digestion just as in the control (lane 1). (B) Trypsin susceptibility assay was further performed with protein substrates including actin (molecular weight = 41.8 kDa), DNase I (30 kDa), the mature form of recombinant invertase (60.6 kDa), proαF (13 kDa), and mitochondrial SOD (32.5 kDa). (C) Oligomeric Aip2p/Dld2p itself does not possess protease activity. Two hundred nanogram of ppαF was incubated with 500 ng of oligomeric Aip2p/Dld2p in the presence of 1 mM ATP for 0–240 min at 30 °C, and the signals were examined by Western blotting with anti-α-factor antibody. (D) The protein conformation modifying activity depends on ATP-binding. The firefly luciferase (300 ng) and oligomeric Aip2p/Dld2p (300 ng) were pre-incubated (final volume of 200 μl) with 1 mM ATP (lane 2), 1 mM ATP with excess amounts (10 mM) of AMP-PNP (lane 4), 1 mM ADP (lane 5) or without nucleotide (lane 3) in buffer A (see Materials and methods) for 15 min at 30 °C. The activity was assayed using the PicaGene luciferase assay kit (Wako Pure Chemicals) in a final volume of 1.2 ml, and the luciferin emission was determined using a luminometer (Stratec Biomedical Systems).

ed'' and adopted an extended globular structure, while the helical tail became longer and thinner (Fig. 3A, Aip2p/Dld2p-HMM). Trypsin susceptibility of HMM also increased after the incubation with oligomeric Aip2p/Dld2p (Fig. 3A).

Surprisingly, even when pathogenic polypeptides such as the rPrP in β-sheet form, α-synuclein or amyloid β (1–42) peptide were tested as substrates in the trypsin susceptibility assay, it was found that trypsin susceptibility increased in the presence of oligomeric Aip2p/Dld2p. Although these pathogenic highly aggregated polypeptides (Fig. 3B) were resistant to 2.5 μg ml^{-1} trypsin, digestion was significant in the presence of only 200 ng ml^{-1} trypsin following incubation with oligomeric Aip2p/Dld2p (Fig. 3B).

Protein conformation modifying activity depends on the growth stage

In order to compare the protein conformation modifying activity in different cell cycles, hexahistidine-tagged oligomeric Aip2p/Dld2p was partially purified from synchronized cells during both log and stationary phases using Ni–NTA–agarose chromatography. The hexahistidine-tagged oligomeric Aip2p/Dld2p was then assayed for its protein conformation modifying activity using F-actin as a substrate. The activity of oligomeric Aip2p/Dld2p purified from log phase cells was almost fivefold greater than that measured in stationary phase cells (Fig. 4). Western blots using anti-Aip2p/Dld2p revealed that Aip2p/Dld2p expression was approximately equivalent in these two phases (Fig. 4), suggesting that the protein conformation modifying activity of oligomeric Aip2p/Dld2p varies with the cell growth stage.

Discussion

The oligomeric Aip2p/Dld2p targeted both properly folded and pathogenic highly aggregated proteins, and exhibited a robust protein conformation modifying activity in vitro. It disrupted the tertiary structure of a variety of properly and stably folded substrate proteins such as the native form of luciferase, actin, HMM, mitochondrial SOD, MDH, and DNase I, as determined in

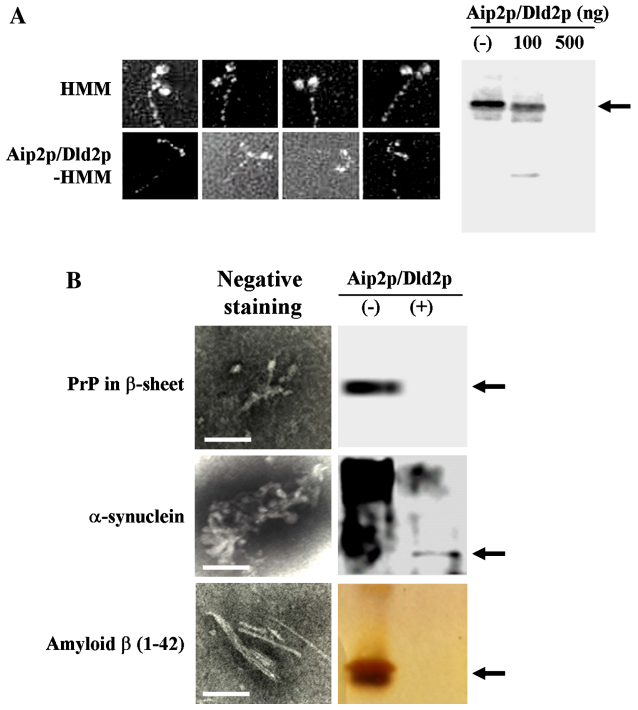

Fig. 3. Oligomeric Aip2p/Dld2p displays broad substrate specificity in vitro. (A) Heavy meromyosin (HMM) is unfolded by oligomeric Aip2p/Dld2p. One hundred microgram per milliliter of HMM was incubated with or without oligomeric Aip2p/Dld2p (100 μg ml^{-1}) in buffer A containing 1 mM ATP as described in Materials and methods. Following incubation, each mixture was subjected to low angle shadowing electron microscopy. Scale bar is 50 nm. Right panel represents the increased trypsin susceptibility of oligomeric Aip2p/Dld2p-treated HMM (100 and 500 ng). HMM was immunostained with anti-HMM polyclonal antibody. (B) Trypsin susceptibility of oligomeric Aip2p/Dld2p-treated pathogenic highly aggregated proteins is dramatically increased. Recombinant prion protein (PrP) in β-sheet form (20 μg), α-synuclein (20 μg), and amyloid β (1–42) peptide (60 μg) were used as specimens for negative staining (left panels). PrP in β-sheet form (300 ng), α-synuclein (200 ng) and amyloid β (1–42) peptide (400 ng) were used for the trypsin susceptibility assay (right panels). PrP and α-synuclein were immunostained with anti-PrP polyclonal antibody K1 (1:200) and anti-α-synuclein antibody, respectively. Amyloid β (1–42) peptide was silver stained according to the manufacturer's instruction (Wako Chemicals). Scale bars are 100 nm.

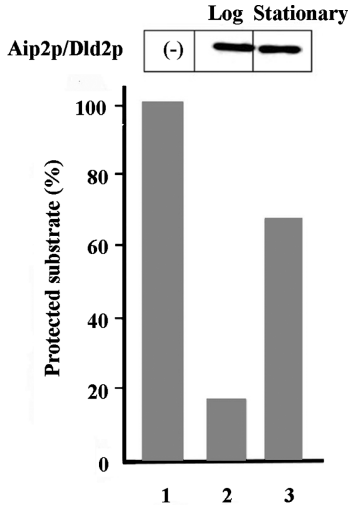

Fig. 4. Aip2p/Dld2p modifies the conformation of F-actin in vivo in a cell cycle-dependent manner. Analysis of protein conformation modifying activity of Aip2p/Dld2p in stationary and log phase yeast cells. Synchronized yeast cells constitutively expressing C-terminally hexahistidine-tagged Aip2p/Dld2p were grown to OD_{600} of 0.5 (log phase, lane 2) or 11 (stationary phase, lane 3) and oligomeric Aip2p/Dld2p was purified from 10 mg each of total yeast cells according to the procedure described in Materials and methods. Protected actin band was detected according to the procedure of trypsin susceptibility assay. A thousandth of purified Aip2p/Dld2p at log or stationary phases was detected by Western blot analysis with anti-Aip2p/Dld2p antibody.

vitro. Both the substrate-binding and protein conformation modifying activities are regulated by the binding of ATP to Aip2p/Dld2p, but not by ATP hydrolysis.

This represents a distinct profile as reflected in known Group I and II chaperonins, which do not target the properly folded proteins [14–17]. In terms of the recognition of native (properly folded) proteins, the folding of native tubulin involves at least seven different chaperone proteins [18], while the structure of the yeast homolog of cofactor A, Rbl2p, is a dimer with largely hydrophilic surfaces, reflecting the fact that it interacts with quasi-native, and not unfolded, β-tubulin. In turn, these chaperone proteins do not recognize misfolded proteins. It is worth noting that the robust protein conformation modifying activity of oligomeric Aip2p/Dld2p modulated the conformation of several pathogenic, highly aggregated proteins such as PrP in β-sheet form associated with prion disease [19], α-synuclein associated with Parkinson's disease [20], and amyloid β (1–42) peptide associated with Alzheimer's disease [21].

As an example of the three-dimensional image of proteins with increased trypsin susceptibility, we directly visualized the "unfolded" structure of substrate protein (HMM) by oligomeric Aip2p/Dld2p with the low angle rotary shadowing electron microscopy. Based on our previous notion that the oligomeric Aip2p/Dld2p exhibits a grapple-like structure of 10–12 subunits with an ATP-dependent opening [4], we are tempted to speculate that substrate proteins probably enter the cavity of oligomeric Aip2p/Dld2p, where they were unfolded in the presence of ATP. Thus, protein unfolding seems to contribute, at least in part, to the aberrant trypsin susceptibility by oligomeric Aip2p/Dld2p.

When the oligomeric Aip2p/Dld2p regulates some protein metabolism in vivo through its unique protein conformation modifying activity, the activity as it is can be extremely dangerous for cells, as it exhibits broad substrate specificity in vitro. Thus, this activity has to be tightly controlled under very stringent regulation such as by other co-factor/s in vivo. In fact, partially purified Aip2p/Dld2p at the log phase possessed higher protein conformation modifying activity and ATP-binding capacity than that of Aip2p/Dld2p purified at the sta-

tionary phase, suggesting the presence of cofactor/s that may provide ATP to oligomeric Aip2p/Dld2p in yeast cells, where F-actin is supposed to be a major target in vivo [5].

Finally, these data further support our previous notion that the oligomeric Aip2p/Dld2p may belong to an unusual class of molecular chaperones [4]. The oligomeric Aip2p/Dld2p represents a unique grapple-like structure in an ATP-dependent opening, and is able to recognize both properly folded and highly aggregated proteins with broad substrate specificity in vitro. Whether these data represent a new regulatory mechanism of protein conformations in vivo has yet to be determined.

Acknowledgments

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