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ORIGINAL PAPER

The microtubule-associated protein, NUD-1, exhibits chaperone activity in vitro

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Abstract Regulation of cell division requires the concerted function of proteins and protein complexes that properly mediate cytoskeletal dynamics. NudC is an evolutionarily conserved protein of undetermined function that associates with microtubules and interacts with several key regulators of mitosis, such as polo-kinase 1 (Plk1) and dynein. NudC is essential for proper mitotic progression, and homologs have been identified in species ranging from fungi to humans. In this paper, we report the characterization of the *Caenorhabditis elegans* NudC homolog, NUD-1, as a protein exhibiting molecular chaperone activity. All NudC/NUD-1 proteins share a conserved p23/HSP20 domain predicted by three-dimensional modeling [Garcia-Ranea, Mirey, Camonis, Valencia, FEBS Lett 529(2–3):162–167, 2002]. We demonstrate that nematode NUD-1 is able to prevent the aggregation of two substrate proteins, citrate synthase (CS) and luciferase, at stoichiometric concentrations. Further, NUD-1 also protects the native state of CS from thermal inactivation by significantly reducing the inactivation rate of this enzyme. To further determine if NUD-1/substrate complexes were productive or simply "dead-end" unfolding intermediates, a luciferase refolding assay was utilized. Following thermal denaturation, rabbit reticulocyte lysate and ATP were added and luciferase activity measured. In the presence of NUD-1, nearly all of the luciferase activity was regained, indicating that unfolded intermediates complexed with NUD-1 could be refolded. These studies represent the first functional evidence for a member of this mitotically essential protein family as having chaperone activity and facilitates elucidation of the role such proteins play in chaperone complexes utilized in cell division. *C. elegans* NUD-1 is a member of an evolutionary conserved protein family of unknown function involved in the regulation of cytoskeletal dynamics. NUD-1 and its mammalian homolog, NudC, function with the dynein motor complex to ensure proper cell division, and knockdown or overexpression of these proteins leads to disruption of mitosis. In this paper, we show that NUD-1 possesses ATP-independent chaperone activity comparable to that of small heat shock proteins and cochaperones and that changes in phosphorylation state functionally alter chaperone activity in a phosphomimetic NUD-1 mutant.

Keywords Chaperone · Dynein · NudC · *nud* · p23 · Plk1

Abbreviations

CS	citrate synthase
GR	glucocorticoid receptor
nud	nuclear distribution
Plk1	polo-kinase I
RRL	rabbit reticulocyte lysate
SEC	size exclusion chromatography
sHsps	small heat shock proteins
TPR	tetracopeptide repeat
UCS	UNC-45/Cro1/She4p

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Introduction

During mitosis, regulatory proteins assist in the synchronization of cellular events via association with chaperones, molecular motors, motor-associated proteins, kinases, and other proteins. Although many of these proteins have been defined, the control of mitotic cell division remains an unresolved problem. Studies of molecular motors and their cargo have provided insights into this mechanism, yet a functional understanding of the mitotic "machinery" remains to be discerned.

Nearly 30 years ago, a genetic screen performed in the fungus, *Aspergillus nidulans*, identified a number of nuclear distribution (*nud*) genes that regulate the movement of nuclei, a fundamental process for growth and development (Morris 1975). Among these, *NudC* was first identified as a mutation that regulated nuclear movement, cell wall deposition, colony growth, and viability (Osmani et al. 1990; Chiu et al. 1997). Other *nud* loci encode either components or proposed regulators of the minus-end-directed microtubule-dependent motor protein dynein and its activator dynactin (Morris 2000). *NudC* is evolutionarily conserved, with homologs identified in many species including *Caenorhabditis elegans*, *Drosophila*, and humans.

Genetic analyses in *A. nidulans* have shown that NudC is functionally linked to the dynein/dynactin motor complex (Morris 2000; Han et al. 2001). Complementary functional studies performed in mammalian cells demonstrated that NudC co-immunoprecipitated with the dynein/dynactin complex (Aumais et al. 2001). An additional NudC homolog, termed NudC-like (NudCL), also binds the dynein complex and appears to be involved in the stabilization of dynein intermediate chain (Zhou et al. 2006). Both mammalian NudC homologs show increased phosphorylation during mitosis (Nishino et al. 2006; Zhou et al. 2006). The kinase responsible for the phosphorylation of NudCL has not yet been identified. However, NudC is known to be phosphorylated at Ser-274 and Ser-326 by the mitotic kinase Plk1 (Zhou et al. 2003). Modulating levels of mammalian NudC via knockdown or overexpression results in mitotic failure and mislocalization of Plk1 (Aumais et al. 2003; Zhou et al. 2003).

We have characterized the sole *C. elegans* NudC homolog, NUD-1, and reported its expression in neurons, multinucleated tissues such as the hypodermis, and in mitotically dividing cells (Dawe et al. 2001). RNAi-mediated knockdown of *nud-1* in *C. elegans* is associated with embryonic lethality resulting from mitotic cell division defects such as misoriented mitotic spindles, an absence of midzone microtubules, and cytokinesis furrow regression (Aumais et al. 2003). Amino acid sequence alignment between NUD-1 and NudC reveals that NUD-1 also contains the same two serine residues noted above; further, knockdown of *nud-1* in *C. elegans* results in mislocalization of PLK-1 whereby it is no longer localized to centrosomes (Faircloth and Caldwell, unpublished observation).

Notably, all homologs of NudC, including NUD-1, contain a domain similar to the human p23 protein and HSP20/alpha-crystallin family of heat shock proteins (Garcia-Ranea et al. 2002). Proteins that contain this p23 core-folding motif often bind to Hsp90 via this region (Zhu and Tytgat 2004; Catlett and Kaplan 2006). This domain was revealed using a combination of three-dimensional and hidden Markov modeling and was identified in several new protein families. One of the new p23-containing proteins identified in that study, Sgt1, a yeast kinetochore assembly protein, has since been functionally validated as an Hsp90 binding partner (Takahashi et al. 2003; Catlett and Kaplan 2006).

The human Hsp90 interactome was recently characterized using proteomic techniques, and NudC was identified as a novel and specific binding partner (Te et al. 2007), thus providing physical evidence for the predicted p23 domain within NudC. In general, p23 proteins and HSP20 family members [often referred to as small heat shock proteins (sHsps)] exhibit ATP-independent chaperone activity and cooperate with ATP-dependent chaperones such as Hsp70 and Hsp90 (Ehrnsperger et al. 1997; Felts and Toft 2003; Haslbeck et al. 2005b).

In this study, we investigate the in vitro chaperone activity of *C. elegans* NUD-1 as a prototypic NudC example of the important, but functionally undefined, NudC protein family. The results of this study reveal that NUD-1 does possess ATP-independent chaperone activity. This was tested via aggregation, inactivation, and refolding assays and NUD-1 was shown to have activity equivalent to or exceeding that of Hsp90.

Materials and methods

Plasmid constructs and site-directed mutagenesis

Whole worm total RNA was extracted as previously described (Locke et al. 2006), and complementary DNA (cDNA) was synthesized using SuperScriptIII (Invitrogen) from an oligo$(dT)_{12–18}$ primer. *nud-1* cDNA was isolated from whole worm cDNA for use in the Gateway Cloning System (Invitrogen) with *Nud-1*GTWY5 and *Nud-1*GTWY3 primers. All primers used are listed in Table 1. Site-directed mutagenesis was performed to change the serine residues at positions 263 and 315 to glutamic acid residues in order to mimic phosphorylation at these sites. Three polymerase chain reactions (PCRs) were performed using *nud-1* cDNA to accomplish these mutations. The first

Table 1 Primers used for cloning and mutagenesis of *C. elegans nud-1* and *pnud-1*

Name	Primer sequence
Nud-1 GTWY 5	ggggacaagtttgtacaaaaaagcaggcttcat gtctcaatatgagcgattcgactcgg
Nud-1 GTWY 3	ggggaccactttgtacaagaaagctgggtttta tccaatttctgaagtccatttcagga
*Nud*263E Fwd	gaagccagagaacgagaaattgagcgacttgg
*Nud*263E Rev	ccaagtcgctcaatttctcgttctctggcttc
mut*nud-1* GTWY 3	Ggggaccactttgtacaagaaagctgggtcttatccaattttagc

Primers are listed in the 5′→3′ direction.

PCR used *Nud-1*GTWY5 and *Nud2*63E Rev primers. *Nud2*63E Fwd and mut*Nud-1*GTWY3 primers were then used to generate the second PCR product. These two PCR products were combined to serve as the template for the third PCR. This reaction used *Nud-1*GTWY5 and mut*Nud-1*GTWY3 primers and resulted in *pnud-1* cDNA, encoding a phosphomimetic NUD-1 protein (pNUD-1). These PCR products were cloned into pDONR221 to create a Gateway entry vector. *nud-1* and *pnud-1* were each introduced into the destination vector pDEST17 which carries an N-terminal His tag. All PCR products and inserts were verified by sequencing (Northwoods DNA, Inc.).

Protein expression/purification

Expression of recombinant NUD-1 and pNUD-1 was carried out in the *Escherichia coli* strain BL21-AI (Invitrogen). Transformed cells were induced with 0.1% arabinose at 37°C for 4 h. Cells were spun down and resuspended in 50 mM sodium phosphate (pH 8.0) containing protease inhibitor cocktail (Roche) and frozen at −80°C. They were then lysed by sonication, and 50% ammonium sulfate was added to the resulting supernatant. Precipitated proteins were pelleted by centrifugation (15,000×*g*) and resuspended in and dialyzed against 50 mM sodium phosphate (pH 8.0). These proteins were purified using HIS-select nickel affinity gel (Sigma) according to the manufacturer's specifications. After purification, the proteins were dialyzed against 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH (pH 7.5), and protein concentrations were determined by bicinchoninic assays using bovine serum albumin (BSA) as a standard.

Materials

Citrate synthase (CS) from porcine heart mitochondria was obtained from Roche and prepared as described previously (Buchner et al. 1998). CS concentration was determined using the published extinction coefficient of 1.78 for a 1 mg/ml solution (Buchner et al. 1998). Luciferase and the luciferase assay system were purchased from Promega. Oxaloacetate, acetyl-CoA, IgG, and dithio-1,4-nitrobenzoic acid were purchased from Sigma and BSA from Invitrogen.

CS inactivation

Thermal inactivation of CS was achieved by incubating the native protein at 43°C. CS (15 μM) was first diluted 1:100 into 40 mM HEPES–KOH (pH 7.5), 0.1 mM EDTA in the presence or absence of additional proteins at 25°C. Inactivation was initiated by shifting the sample to 43°C. Aliquots were taken at specific time points, and CS activity was measured according to Srere et al. (1963).

Aggregation assays

To measure CS aggregation, CS (15 μM) was diluted 1:100 in 40 mM HEPES–KOH (pH 7.5) and equilibrated at 43°C in the presence and absence of chaperones or controls. All NUD-1 and pNUD-1 molar concentrations were based on the octameric form. Luciferase (10 μM) was diluted as above and equilibrated at 42°C with and without chaperones or controls. To monitor kinetics of thermal aggregation, light scattering was measured in a PerkinElmer LS50-B luminescence spectrophotometer at 370 nm with a spectral bandwidth of 2.5 nm.

NUD-1 analysis by SEC

Purified NUD-1 and pNUD-1 protein samples were spun at 4°C for 10 min at 16,000×*g* and filtered through a 0.45-μm syringe filter. Samples were analyzed on a Superose 6 10/300 GL column (GE Healthcare; 10×300 mm; separation range of 5–5,000 kDa) with a mobile phase consisting of 100 mM HEPES–KOH (pH 7.5), 150 mM KCl, running at 0.5 ml/min at 22°C. Proteins were detected by absorbance at 280 nm.

Luciferase refolding assay

Luciferase refolding assays were performed as described by Lee et al. (1997) and as modified by Abdulle et al. (2002). Luciferase (0.2 μM) was incubated with various concentrations of NUD-1, pNUD-1, or IgG in refolding buffer [5 mM $MgCl_2$, 10 mM KCl, 50 mM HEPES–KOH (pH 7.5), and 2 mM DTT] at 22°C or 42°C for 15 min. To prevent luciferase adsorption to walls, tubes were treated with 1 mg/ml BSA for 15 min then rinsed with water. Ten microliters of the mixtures was added to a solution pre-incubated at 30°C containing 18 μl of rabbit reticulocyte lysate (RRL; Promega) and 2 μl of 0.1 M ATP. At various times, 2 μl of the sample was removed, added to 48 μl of

50 mM HEPES–KOH (pH 7.5), vortexed, and diluted 10-fold into luciferase assay reagent (Promega). Luciferase activity was measured with a Promega GloMAX 20/20 luminometer.

Statistics

Statistical analysis for chaperone activity was performed using the Student's *t* test to compare NUD-1 with pNUD-1 endpoint results in various assays. Significance was determined as $P<0.05$.

Results

Production and analysis of recombinant NUD-1 and pNUD-1

The *nud-1* cDNA was fused to a 6X histidine tag at the N terminus and expressed in bacteria as a full-length 6X-HIS-NUD-1 protein. It was purified over a nickel column and will be referred to hereafter as NUD-1 (Fig. 1). Notably, mammalian NudC is phosphorylated at two highly conserved serine residues by Plk1; the phosphorylated form of NudC is biologically active during cell division (Zhou et al. 2003). These conserved sites are also present in the *C. elegans* homolog, NUD-1, at residues 263 and 315. Since phosphorylation is known to have an effect on sHsps, it was important that we investigate the role of phosphorylation on the activity of NUD-1. To do so, we constructed a mutant to mimic phosphorylation at these sites by using site-directed mutagenesis to change the conserved serines to glutamic acid residues. The resulting mutant phosphomimetic protein, pNUD-1, was also produced and purified as a 6X-HIS fusion protein (Fig. 1).

Many sHsps are known to form large oligomeric complexes whose composition can be altered when phosphorylated (Haslbeck et al. 2005b). Since NUD-1 is well within the size range of the sHsps at 37 kDa, we wanted to know if it was capable of forming a larger complex and, if so, to determine if its size could be altered by phosphorylation. Therefore, we utilized size exclusion chromatography (SEC) to analyze the oligomeric state of NUD-1 as well as pNUD-1. NUD-1 forms a large oligomer (~300 kDa) consisting of eight NUD-1 monomers (Fig. 2). It is also evident that phosphorylation has no effect on the oligomeric state of NUD-1 since there is no shift in molecular weight observed in the pNUD-1 mutant (Fig. 2). For all subsequent assays, concentrations of NUD-1 and pNUD-1 were calculated using the octameric form.

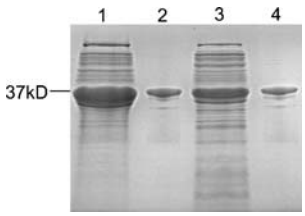

Fig. 1 Expression and purification of NUD-1 and pNUD-1. Crude extract from *E. coli* BL21-AI cells containing the pDEST17 expression vector with either NUD-1 (lane *1*) or pNUD-1 (lane *3*) was collected after 4 h of arabinose induction. NUD-1 (lane *2*) and pNUD-1 (lane *4*) were purified by nickel affinity column chromatography and analyzed by gel electrophoresis with Coomassie staining

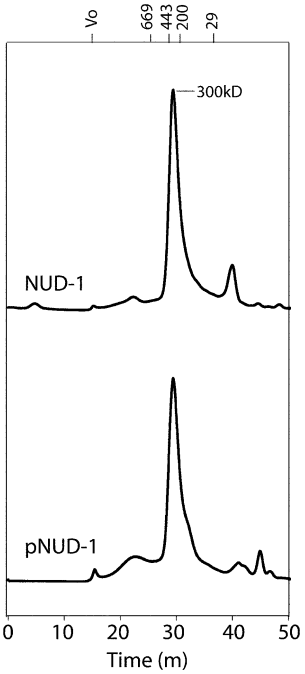

Fig. 2 NUD-1 and pNUD-1 form large oligomeric complexes. To analyze oligomerization state, both NUD-1 and pNUD-1 (2 μM) were run on a Superose 6 10/300 GL column (GE Healthcare) as described in "Materials and methods"

NUD-1 suppresses aggregation of CS and luciferase

Since many of the proteins that contain the p23 domain have been found to have in vitro chaperone activity, we sought to determine if NUD-1 and pNUD-1 also exhibited such activity. We used CS and luciferase as substrates in a common assay which examines the ability of a chaperone to inhibit aggregation (Jakob et al. 1995; Herbst et al. 1997; Morrow et al. 2006). Both substrates aggregate at high temperatures (>40°C) and aggregation is monitored by light scattering.

Figure 3 shows the thermal aggregation of CS after incubation at 43°C over 20 min. At equimolar concentrations of NUD-1 (1×), CS aggregation was reduced by 93.5±3.0% (Fig. 3A). This high level of protection was even maintained when the concentration of NUD-1 was decreased fourfold (0.25×). This nearly absolute protection can be compared to excess BSA, a negative protein control, which still shows high levels of aggregation. To determine the potential for phosphorylation to affect chaperone activity, pNUD-1 was tested as well (Fig. 3B). Imitating

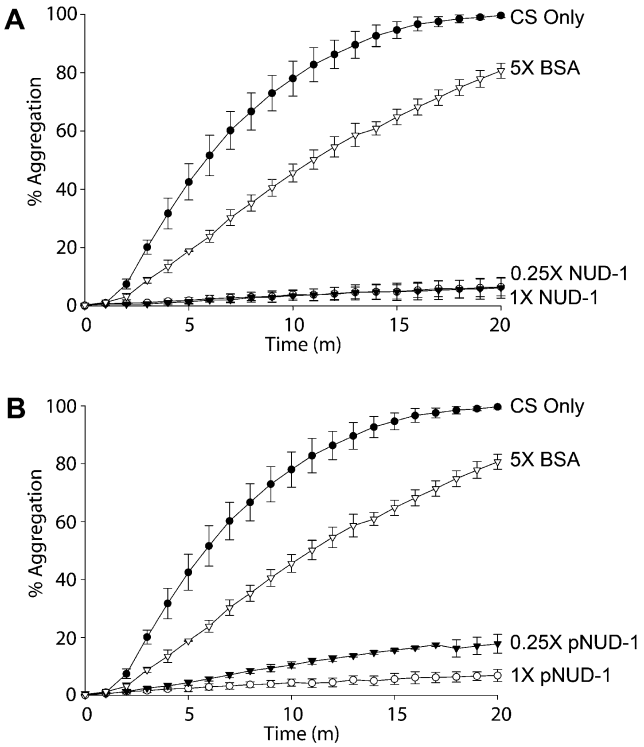

Fig. 3 NUD-1 and pNUD-1 inhibit heat-induced aggregation of citrate synthase (*CS*). CS (0.15 μM) was incubated at 43°C for 20 min either alone (*filled circle*), in the presence of BSA (0.75 μM, *triangle*), NUD-1 (0.15 μM, *open circle*; 0.0375 μM, *filled inverted triangle*) in **A**, pNUD-1 (0.15 μM, *open circle*; 0.0375 μM, *filled inverted triangle*) in **B**. Aggregation was determined by light scattering at 370 nm. Data are representative of three trials and were calculated as a percentage of the maximum aggregation of CS after 20 min for each trial and are expressed as the mean±SD

phosphorylation in this manner had no effect (P=0.88) on the chaperone activity of NUD-1 at the 1× concentration (93.2±2.0%). However, at the lower concentration, the chaperone activity of pNUD-1 was significantly lower than NUD-1 (82.2±3.2%; P=0.01).

To demonstrate that NUD-1 chaperone activity exhibits general substrate specificity, we verified that the protein was also able to inhibit thermal aggregation of luciferase. Figure 4 shows the aggregation of luciferase after incubation at 42°C over 20 min. Similar to its effect on CS, an equimolar amount of NUD-1 decreased aggregation by 98±1.4% (Fig. 4A). A reduced concentration of NUD-1 was also effective with 87.1±4.8% protection. Incubation with pNUD-1 exhibited a similar result (Fig. 4B). At an equimolar concentration, aggregation was decreased by 94.2±4.22% and was not significantly different from NUD-1 (P=0.21). However, at the lower concentration, aggregation was reduced by 69.9±5.3%, which is significantly lower than with NUD-1 (P=0.01). These data correspond well with the CS aggregation data and together demonstrate that phosphorylation has a significant effect on the ability of NUD-1 to prevent aggregation. NUD-1 and pNUD-1, at stoichiometric concentrations, are both capable of nearly complete suppression of aggregation, but at substoichiometric concentrations, NUD-1 exhibits greater chaperone activity than pNUD-1.

Thermal inactivation of CS

Although NUD-1 can prevent protein aggregation, it is not known whether the protected proteins retain activity. To further characterize the interactions of NUD-1 with unfolded proteins, we examined the thermal inactivation of CS. This assay measures the amount of CS activity remaining after exposure to heat-denaturing conditions in the presence of an additional chaperone. For example, in the presence of excess IgG (a negative control), CS rapidly lost its activity after incubation at 43°C, decreasing to less than 10% after only 5 min (Fig. 5). This inactivation reaction followed first-order kinetics with an inactivation rate of 19.2×10^{-3} s^{-1} (Table 2). In contrast, an eightfold molar excess of NUD-1 decreased the inactivation rate by 88%; this is similar to the effect of Hsp90 at the same molar ratio (Jakob et al. 1995).

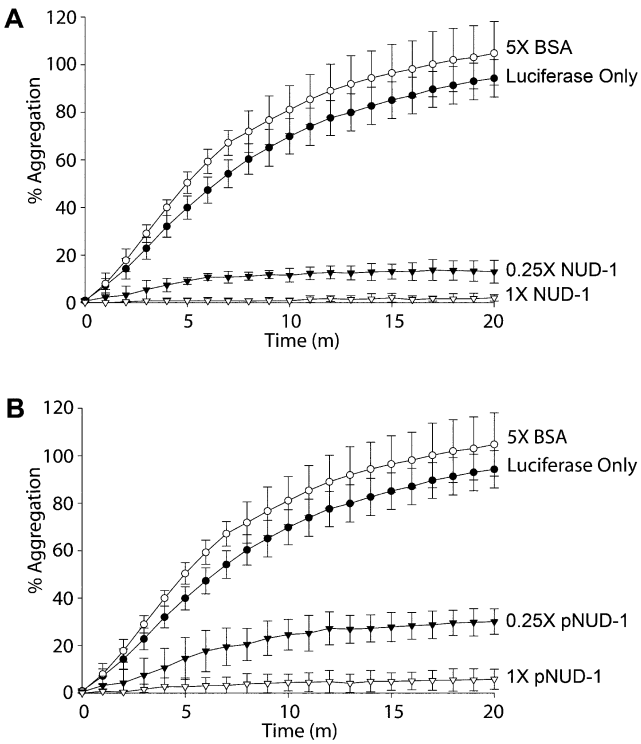

Fig. 4 NUD-1 and pNUD-1 inhibit heat-induced aggregation of luciferase. Luciferase (0.1 μM) was incubated at 42°C for 20 min either alone (*filled circle*), in the presence of BSA (0.75 μM,), NUD-1 (0.1 μM, *triangle*; 0.025 μM, *filled inverted triangle*) in **A**, or pNUD-1 (0.1 μM, *triangle*; 0.025 μM, *filled inverted triangle*) in **B**. Aggregation was determined by light scattering at 370 nm. Data are representative of three trials and were calculated as a percentage of the maximum aggregation of luciferase after 20 min for each trial and are expressed as the mean±SD

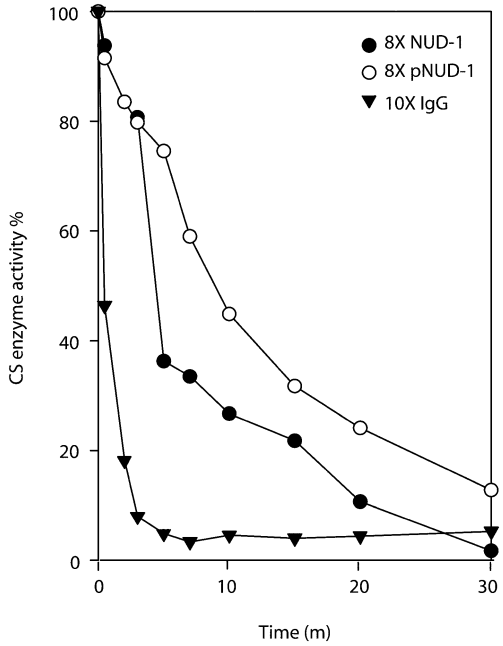

Fig. 5 NUD-1 and pNUD-1 decrease the rate of heat-induced inactivation of CS. CS (0.15 μM) was incubated for 30 min in the presence of 1.5 μM IgG (*filled inverted triangle*), 1.2 μM NUD-1 (*filled circle*), or 1.2 μM pNUD-1 (*open circle*), and CS activity was measured at various time points. The enzymatic activity of CS was expressed as a percentage of initial CS activity

The presence of pNUD-1 resulted in even greater thermoprotection with a 94% decrease in the inactivation rate. These data demonstrate that both NUD-1 and pNUD-1 are able to bind to and stabilize early folding intermediates of CS under thermal denaturing conditions.

NUD-1 can maintain heat-denatured luciferase in a folding competent state

To verify that the partially unfolded proteins trapped by these chaperones were in a refoldable state, an in vitro luciferase refolding assay was employed. In this assay, luciferase is heat-denatured in the presence or absence of chaperones and then allowed to refold at a lower temperature in the presence of ATP and other chaperone machinery present in RRL. Luciferase maintained at 25°C showed a slight increase in activity to 102% after the addition of RRL, but was stable throughout the refolding period (active luciferase, Fig. 6). However, when luciferase was incubated at 42°C for 15 min in the absence of any chaperones, or with excess IgG, the activity decreased to less than 10% and no recovery of activity after the refolding period was observed (Fig. 6A, B). In the presence of an equimolar concentration of NUD-1, 88.2±12.2% luciferase activity was regained; doubling the concentration of NUD-1 did not alter the activity recovered (Fig. 6A). When luciferase was denatured in the presence of an equimolar concentration of pNUD-1, 54.7±8.0% activity was regained and, as with NUD-1, an increase in pNUD-1 concentration had no further effect (Fig. 6B). However, there was a significant difference in chaperone activity between NUD-1 and pNUD-1 at both concentrations (1×, P=0.02; 2×, P=0.04). From these data, it is clear that both NUD-1 and pNUD-1 can maintain heat-denatured lucifer-

Table 2 Influence of various proteins on the rate of thermal inactivation of CS at 43°C

Addition to inactivation buffer	Rate constant ($\times 10^{-3}$ s^{-1}) of CS thermal inactivation[a]
1.50 μM IgG	19.2
1.20 μM NUD-1	2.3
1.20 μM pNUD-1	1.2

[a] Rate constants were obtained from the inactivation kinetics shown in Fig. 5A.

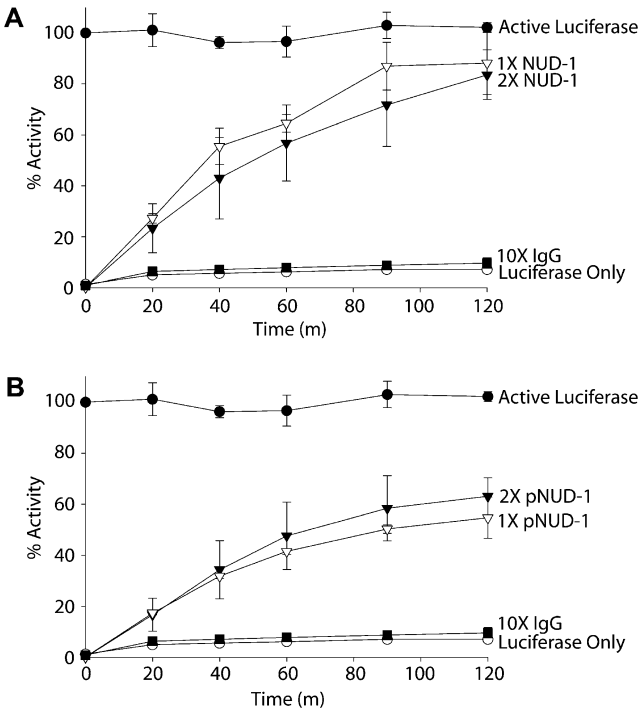

Fig. 6 Luciferase can be refolded in the presence of either NUD-1 or pNUD-1. Active luciferase (0.2 μM, *filled circle*) was measured after incubation at 25°C for the indicated times. All other measurements were made after incubation at 42°C for 15 min. Luciferase (0.2 μM) was incubated either alone (*open circle*) or in the presence of IgG (2 μM, *filled square*). **A** Luciferase was incubated with NUD-1 (0.2 μM, *triangle*; 0.4 μM, *filled inverted triangle*). **B** Luciferase was incubated with pNUD-1 (0.2 μM, *triangle*; 0.4 μM, *filled inverted triangle*). The refolding step was performed at 30°C for 120 min in rabbit reticulocyte lysate (*RRL*) supplemented with ATP, and luciferase activity was determined at the different time points. Data are representative of three trials and are presented as percentage of luciferase activity after 15 min of incubation at 25°C. Data are expressed as mean±SD

ase in a refoldable state that allows it to be refolded by other chaperones into a fully active enzyme, but NUD-1 exhibits greater chaperone activity.

Discussion

We describe the previously unreported ability of NUD-1 to function as a molecular chaperone, with multiple substrates and across a variety of established assays. The purified protein forms a large oligomer (>300 kDa), corresponding to an octamer of NUD-1 subunits; this is characteristic of chaperones in the sHsp family. One of the features of some sHsps is that their oligomeric state can be altered by phosphorylation, sometimes changing chaperone activity levels (Sun and MacRae 2005). Thus, we also used molecular mimicry of serine phosphorylation of NUD-1 by substituting glutamate to study its effects on oligomerization and chaperone activity (Eidenmuller et al. 2000; Longshaw et al. 2004).

The ability to inhibit aggregation of heat-denatured proteins is a characteristic of most molecular chaperones (Borges and Ramos 2005). Both NUD-1 and pNUD-1 prevented more than 90% of the aggregation of CS and luciferase at equimolar concentrations. This is similar to the high activity of sHsps, which is due to their high substrate binding affinity (Lee et al. 1995; Koteiche and McHaourab 2003). The decrease in chaperone activity due to phosphorylation is also seen with the sHSPs (Rogalla et al. 1999; Ito et al. 2001).

We further examined NUD-1 chaperone activity using a CS inactivation assay. In this context, NUD-1 protects CS from thermal denaturation by slowing the rate of inactivation by more than 80%. This result is suggestive of a transient interaction between NUD-1 and early folding CS intermediates (Jakob et al. 1995). Notably, the cochaperone p23 has also been shown to reduce the inactivation rate of CS (Bose et al. 1996). In contrast to NUD-1 and p23, sHsps such as Hsp25 and Hsp30C appear to form a more long-lived complex with CS intermediates (Ehrnsperger et al. 1997; Fernando and Heikkila 2000) and are therefore unable to prevent CS inactivation (Ehrnsperger et al. 1997). Due to the tight binding of sHsps to unfolded substrates, they require the addition of an ATP-dependent chaperone, such as Hsp70, and more permissive conditions before substrates can be released (Ehrnsperger et al. 1997; Lee et al. 1997; Haslbeck et al. 2005a).

Using a luciferase refolding assay in the presence of equimolar NUD-1, we determined that NUD-1 was able to maintain luciferase in a folding-competent state; after the addition of RRL and ATP, ~90% of the luciferase activity was recovered. This has also been demonstrated for many sHsps (Bose et al. 1996; Fernando and Heikkila 2000; Abdulle et al. 2002). Notably, while pNUD-1 displayed high chaperone activity in this assay, it was significantly lower than NUD-1, demonstrating regulation of NUD-1 chaperone activity by phosphorylation.

Several years ago, NUD-1 was predicted to contain a p23/HSP20 domain via three-dimensional modeling programs (Garcia-Ranea et al. 2002). It is interesting to note that while our assays indicate that NUD-1 shares many characteristics with proteins in the sHsp family, there are distinct differences in activity and it does not fit the exemplar profile of either a sHsp or a p23-like protein exclusively. These proteins predominantly consist of the p23/Hsp20 domain with no other known functional domains present (Weikl et al. 1999; Stromer et al. 2003). However, within NUD-1, the p23/HSP20 domain is fused with additional domains that, together, likely provide the proper three-dimensional structure required for NUD-1/NudC activity (Garcia-Ranea et al. 2002). In metazoans, NudC homologs have an N-terminal extension consisting of two coiled-coil domains; further, these proteins share a C-terminal region that is phosphorylated by, and binds to, Plk1. The p23/Hsp20 domain is located between these two domains (see Fig. 7). Therefore, it might not be surprising that NUD-1 appears to have characteristics of multiple chaperone types whereby it prevents aggregation and forms oligomers similar to sHsps (instead of monomers like p23), but displays thermal inactivation properties more similar to p23 (and unlike sHsps). This distinct combination of observed chaperone traits may reflect the multidomain architecture of NUD-1.

A greater understanding of NUD-1 function requires further investigation into these unique chaperone properties. Since it contains a p23 domain, which is known to bind Hsp90 (Felts and Toft 2003; Lee et al. 2004; Wu et al. 2005), the relationship between NUD-1 and Hsp90 could be investigated to determine if it acts as a cochaperone. Classification of cochaperones has been traditionally based upon secondary structural domains. However, considering that NUD-1 was identified as a p23 domain-containing protein based on three-dimensional modeling, it does not conform to families defined purely by their secondary structure. Thus, functional studies of NUD-1 cochaperone activity with Hsp90 are still warranted. While beyond the

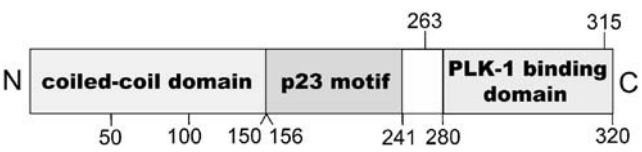

Fig. 7 Schematic representation of the three primary domains identified within NUD-1. The positions of the two conserved *C. elegans* serine residues altered to mimic PLK-1 phosphorylation are indicated on *upper portion* of the figure (amino acids 263 and 315)

scope of this study, the binding of NUD-1 to Hsp90 could be confirmed by SEC and/or co-immunoprecipitation experiments. Structure–function studies involving removal of all or part of the p23 domain to examine the specificity of binding and characterization of the ability of NUD-1 to modulate the ATPase activity of Hsp90 could potentially advance our understanding of both these proteins. Furthermore, it would be interesting to determine if the N- or C-terminal regions of NUD-1, involved in protein–protein interactions or PLK-1 binding, respectively, are necessary for chaperone activity. Previous studies with p23 have shown that its unstructured C terminus is responsible for its chaperone activity (Weikl et al. 1999).

Many chaperones interact with Hsp90 through two well-characterized Hsp90-binding domains, the p23/HSP20 and tetracopeptide repeat (TPR) domains (Lamb et al. 1995; Blatch and Lassle 1999). Glucocorticoid receptor (GR) transport to the nucleus is a well-characterized function of Hsp90 that requires association with an immunophilin through its TPR domain. In this regard, the GR/Hsp90 complex is linked to the immunophilin, which then attaches to the dynein complex for retrograde movement along microtubules. This interaction of a chaperone with a cytoskeletal element is not unique to this class of chaperones. Additional chaperones interact with motor proteins and cytoskeletal elements; these include the sHsps, which interact with actin filaments and tubulin (Gusev et al. 2002; Mitra et al. 2007), the UNC-45/Cro1/She4p (UCS) family of chaperones that interact with myosin (Hutagalung et al. 2002), and a plethora of tubulin chaperones, which include cofactors and chaperonins (Lewis et al. 1997; Lundin et al. 2008).

Studies in *A. nidulans* and mammalian cells have shown that NudC is associated with the microtubule minus-end directed dynein/dynactin motor complex (Morris 2000; Aumais et al. 2001; Han et al. 2001). This complex may target regulatory and effector molecules to the midzone during the cytokinesis phase of mitosis. When NudC is knocked down using small interfering RNA, cytokinesis defects result, and Plk1 is no longer localized at the centrosomes during metaphase or at the midbody during cytokinesis (Aumais et al. 2003; Zhou et al. 2003). These cytokinesis defects can be partially rescued by ectopic expression of wild-type NudC, but not by expression of NudC with mutations in the Plk1 phosphorylation sites (Zhou et al. 2003). Furthermore, when NudC-deficient cells are reconstituted with wild-type NudC, proper Plk1 localization is restored, but not when phospho-defective NudC is used (Nishino et al. 2006).

Notably, in our study, we have discovered that NUD-1 chaperone activity levels can be regulated by phosphorylation. In the context of these findings, the localization and activity of NudC/NUD-1 may be dissociated. However, the cellular dysfunction associated with aberrant phosphorylation of NudC by Plk1 may be a consequence of mislocalization as well as chaperone activity. The chaperone activity of NUD-1/pNUD-1 may maintain the functionality of proteins, perhaps even Plk1, as they traverse the cellular milieu.

The interaction of NudC with molecular motor proteins, such as dynein, as well as Plk1, underscores the pivotal role NudC plays in cell division. We speculate that NudC and its various homologs may function in the capacity of a chaperone to correctly localize mitotic proteins, perhaps via an Hsp90 and/or dynein-mediated mechanism.

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