Cdc48 can distinguish between native and non-native proteins in the absence of cofactors

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Abstract The ATPase Cdc48 is required for membrane fusion and protein degradation. Recently it has been suggested that Cdc48 in a complex with Ufd1 and Npl4 acts as an ubiquitin-dependent chaperone. Here it is shown that recombinant Cdc48 alone can distinguish between the native and the non-native conformation of model substrates. First, Cdc48 prevents luciferase from aggregating following a heat shock. Second, it inhibits the aggregation of rhodanese upon dilution. Third, Cdc48 binds specifically to heat-denatured luciferase. These chaperone-like functions seem to be independent of ATPase activity. Furthermore, Cdc48 can act as a co-chaperone in the Hsc70–Hsp40 chaperone system. These results show that Cdc48 possesses chaperone-like activities and can functionally interact with Hsc70. Cdc48's ability to recognise denatured proteins can also be a source of unspecific binding in biochemical interaction experiments.

Key words: Cdc48; p97; VCP; ATPase associated with various cellular activities; Chaperone; Ubiquitin; ER-associated protein; Hsp70

1. Introduction

Yeast Cdc48 and its mammalian homologue p97/VCP are AAA proteins (ATPases associated with various cellular activities) that appear in an astonishing variety of cellular and biochemical contexts: cell cycle progression [1], ER membrane fusion [2], transitional ER assembly [3], nuclear fusion [4], Golgi reassembly [5,6], degradation of ubiquitin fusion proteins and of IκBα [7–9], transcription factor processing [10,11], degradation of ER-associated proteins (ERAD) [12–16], and apoptosis [17,18]. It has been suggested that the unifying principle of the diverse functions of the AAA proteins is their chaperone function [19]. Chaperones specifically bind proteins in a certain folding state (e.g. in the unfolded native conformation) and support the folding or unfolding of proteins [20]. The chaperone-like activity of Cdc48/p97 would be required to disassemble SNARE complexes in membrane fusion, as well as for recognising proteins that are to be processed or degraded after their release from the ER. Here it is shown by four criteria that Cdc48 possesses chaperone-like activity.

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2. Materials and methods

2.1. Cloning, expression and purification of maltose binding protein (MBP)–Cdc48

Cdc48 was expressed with the BacculoGold transfection kit (Pharmingen). CDC48 was PCR-amplified from yeast genomic DNA (using primers that introduce a *Pst*I site at the 5′-end and a *Bam*HI site at the 3′-end) and cloned into pVL1392 (Pharmingen). The MBP epitope (from pMALc2, New England Biolabs) was inserted into the *Bam*HI site. Sf9 cells were transfected and the virus titre was raised through three passages. For expression of Cdc48, cells were incubated at 28°C in liquid culture. Cells were harvested 3 days after infection (1000×*g*, 5 min, room temperature), washed in phosphate-buffered saline (PBS) and resuspended in buffer L (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 0.1 mM ATP) with complete protease inhibitor cocktail (Roche). Cells were lysed by 10 strokes in a Dounce homogeniser and centrifuged (20 000×*g*, 20 min, 4°C). Recombinant protein was bound to amylose-Sepharose (New England Biolabs), unspecifically bound proteins were removed by washes with buffer L. Cdc48 was eluted with 10 mM maltose in buffer L. The protein was frozen in liquid nitrogen and stored at −75°C. Purity of the recombinant protein, estimated after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), was greater than 95%. The control protein was prepared in the same manner using a construct expressing MBP without Cdc48. Alternatively MBP was purchased (MBP2*; New England Biolabs) and dialysed against buffer L. MBP and MBP2* behaved indistinguishably in the assays described in this work. ATPase activity was determined as described [21].

2.2. Chaperone assays

For luciferase aggregation assays sample proteins were incubated in buffer A (20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM ATP) including 0.5 μM firefly luciferase (Promega). Samples were kept in ice for 5 min, then transferred to 42°C for 15 min. After centrifugation (20 min at 100 000×*g*) pellets were analysed by SDS–PAGE and Western blotting. Blots were probed with anti-luciferase antibodies (Promega).

For the rhodanese aggregation assay 500 nM rhodanese (Sigma) was preincubated for 1 h at 25°C in 40 mM Tris–HCl, pH 7.4, 6.5 M guanidinium hydrochloride, 8 mM DTT. The denatured rhodanese was diluted 1:300 into 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP including the sample proteins. Subsequently absorption was determined at 320 nm over a course of 10 min.

For analysis of Cdc48/luciferase complexes firefly luciferase was kept at 42°C or on ice in buffer A in the absence or presence of Cdc48. After 15 min samples were transferred to ice, 5% glycerol was added and the samples were centrifuged for 20 min at 150 000×*g* (TLA 100, 4500 rpm). The supernatants were analysed by gel exclusion chromatography on a SMART Superose 6 column (Pharmacia) at 4°C. Fractions were separated by SDS–PAGE and Western blotting. Blots were decorated with anti-MBP antibodies (New England Biolabs) and anti-luciferase antibodies.

Co-chaperone activity of Cdc48 in Hsc70–Hsp40-mediated refolding of luciferase was carried out essentially as described [22]. Cdc48 was incubated in buffer R (10 mM MOPS–KOH, pH 7.2, 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 2 mM ATP) with 3 μM Hsc70 and 2 μM Hsp40 (a gift from J. Höhfeld) for 5 min at 25°C in a total

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volume of 18 μl. Firefly luciferase was added in a volume of 2 μl at a final concentration of 60 nM. The luciferase was thermally denatured by incubation at 42°C for 15 min. Samples were then incubated at 30°C. At the indicated time points, 1 μl aliquots were analysed for luciferase activity with the luciferase assay (Promega) using a Berthold Lumat LB9501 luminometer.

3. Results

3.1. Expression of Cdc48 in Sf9 insect cells

Attempts to obtain functional recombinant Cdc48 from *Escherichia coli* were not successful. Therefore Cdc48 was expressed with a MBP tag in an eukaryotic expression system based on bacculovirus-transformed Sf9 cells. The protein was purified with amylose-Sepharose without the use of detergent or glycerol. ATPase activity of the purified protein was determined to be 650 pmol ATP/μg fusion protein/min (equivalent to 938 pmol/μg/min for the untagged Cdc48). Published values range between 720 and 1560 pmol/μg/min [23,24], depending on the source, the purity (including cofactors), the buffer and the temperature. For comparison, the ATPase activity of NSF (without cofactors) is about 66 pmol/μg/min.

3.2. Cdc48 reduces luciferase precipitation during heat shock

Many chaperones can stabilise certain folding states in solution and thereby prevent proteins from aggregating or even support their refolding. Incubation of luciferase at 42°C leads to its denaturation and precipitation. A chaperone should be able to prevent aggregation following denaturation. If Cdc48 is present during thermal inactivation of the luciferase (conditions, which do not effect the activity of Cdc48 itself [23]), precipitation of luciferase following heat shock can be prevented (Fig. 1, column 3). The control protein MBP does not stabilise luciferase (Fig. 1, column 2). Cdc48 can also prevent the aggregation of luciferase when ADP (Fig. 1, column 4) replaces ATP in the denaturation buffer, or when nucleotide cycling is abolished by the addition of EDTA (column 5).

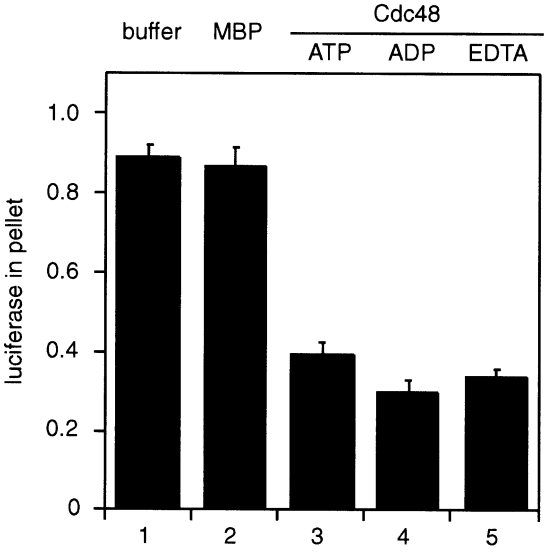

Fig. 1. Cdc48 suppresses the aggregation of luciferase upon heat shock. Firefly luciferase was thermally denatured in the presence of buffer alone (1), 1 μM MBP (2), or 1 μM Cdc48 with 1 mM ATP (3), 1 mM ADP (4) or 10 mM EDTA (5). Following high-speed centrifugation the amount of precipitable luciferase was determined densitometrically after SDS–PAGE and Western blotting. Luciferase concentration was 0.5 μM. Luciferase input is set to 1.

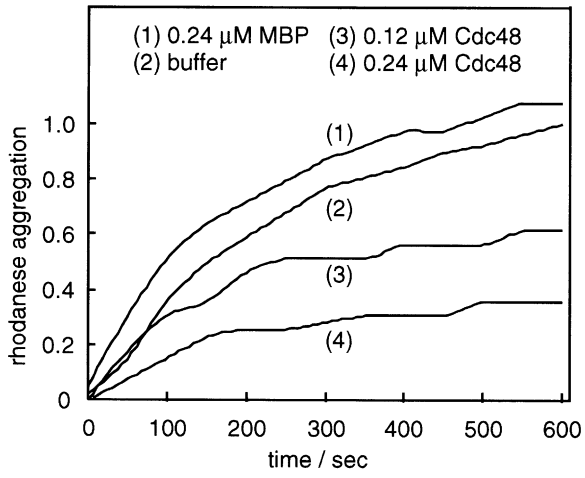

Fig. 2. Cdc48 can reduce the aggregation of denatured luciferase upon dilution. Rhodanese was denatured in guanidinium hydrochloride buffer and diluted 300-fold in the presence of buffer only (2), MBP (1), or Cdc48 (3 and 4). Aggregation of rhodanese was then monitored photometrically. The buffer only signal after 10 min was set to 1. Rhodanese concentration was 0.5 μM.

3.3. Cdc48 reduces aggregation of denatured rhodanese upon dilution

To test if this function is independent of luciferase, an assay with the heterologous substrate rhodanese was used. In this assay the denaturation step can be separated from the aggregation step of the test substrate. Rhodanese is denatured in 6 M guanidinium chloride at 25°C. Dilution of rhodanese in buffer alone leads to its aggregation. Addition of Cdc48 prior to dilution strongly suppresses aggregation (Fig. 2). Cdc48 at a concentration as low as 240 nM reduces the rhodanese aggregation by 65%. The control protein MBP does not suppress the aggregation of rhodanese. Cdc48's ability to suppress rhodanese aggregation is not abolished when ATP cycling is prevented by the addition of EDTA (not shown).

3.4. Cdc48 specifically binds denatured luciferase

One of the hallmarks of chaperones is their ability to specifically bind unfolded proteins. To confirm that the effect shown in Fig. 2 is indeed due to Cdc48's ability to specifically interact with denatured proteins, luciferase was denatured in the presence or absence of Cdc48. Protein aggregates were removed by centrifugation and the soluble supernatant was analysed by gel filtration. If Cdc48 is present during luciferase denaturation, luciferase is found together with Cdc48 in high molecular weight fractions between 2000 and 400 kDa (Fig. 3, bottom two panels). For comparison, the non-denatured luciferase can be detected at the size of the monomeric luciferase of about 66 kDa (Fig. 3, first panel). Upon heat denaturation of luciferase in the absence of Cdc48, no luciferase is found in the supernatant after high-speed centrifugation (Fig. 3, second panel). After heat shock, Cdc48 alone remains soluble and appears in fractions of high molecular mass, corresponding to its hexameric structure. In the presence of luciferase, Cdc48 is shifted to even higher molecular mass fractions, indicating that a substantial amount of luciferase binds to one

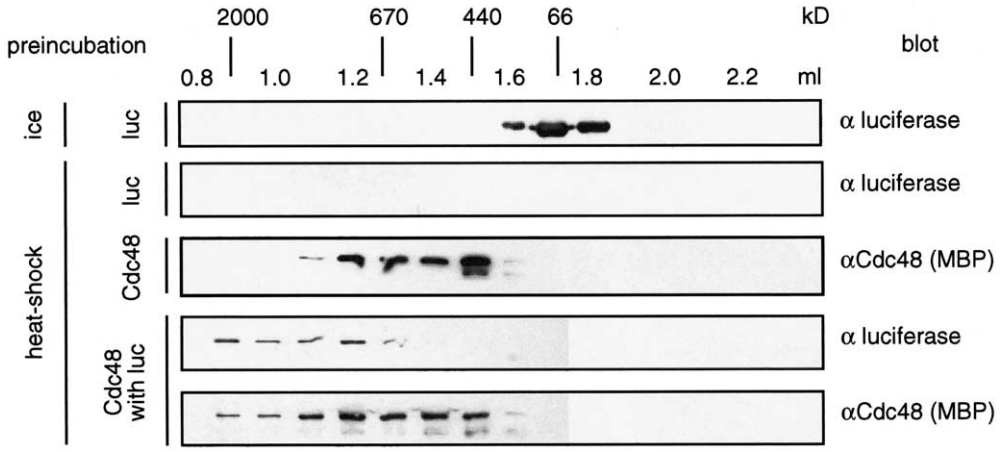

Fig. 3. Cdc48 specifically binds to denatured firefly luciferase. Luciferase and Cdc48 were incubated alone or together at 42°C or on ice. Supernatants were analysed by size exclusion chromatography on a gel filtration column (exclusion limit 2000 kDa), SDS–PAGE and Western blotting. Cdc48 and luciferase concentrations were 1 μM each. The molecular weight standards used for the gel filtration are DEAE-Dextran (approximately 2000 kDa), thyroglobulin (670 kDa), ferritin (440 kDa), and bovine serum albumin (66 kDa).

hexamer of Cdc48. About 18 molecules of luciferase would have to bind to a Cdc48 hexamer, to show such a molecular shift. Alternatively, luciferase molecules bridge two Cdc48 hexamers and thereby cause the appearance of dodecamers of double molecular weight.

Chaperones that (independently of ATP) bind denatured proteins thereby exert a 'holding' function for subsequent 'folding' steps [25]. For example Hsp104 is regarded as such a holding device for the Hsc70–Hsp40 chaperone system [26]. It is assumed that these (partially unidentified) 'holding' factors are present in the reticulocyte lysate, which is required in the 'classical' chaperone system consisting of the ATPase Hsc70 and the chaperonin Hsp40 [22].

3.5. *Cdc48 can replace the cytosol requirement in the Hsc70–Hsp40 chaperone system*

Cdc48 can also support the refolding of heat-denatured luciferase similar to Hsp104. Luciferase was denatured and incubated in the presence of Hsc70–Hsp40 and either buffer alone, MBP, or Cdc48. Luciferase activity was determined over a period of 1 h. The addition of reticulocyte lysate (RL) leads to 84% recovery of the luciferase activity after 1 h (Fig. 4). Cdc48 can replace reticulocyte lysate and restore the activity of the Hsc70–Hsp40 system to 74%, whereas MBP gives only 38% recovery of the luciferase activity. Thus 1.5 μM Cdc48 in the presence of 3 μM Hsc70 and 2 μM Hsp40 can substitute for 84% of the 'co-chaperone' effect obtained with 2.5% reticulocyte lysate (relative to the effect of MBP), whereas Cdc48 alone does not refold luciferase (not shown).

4. Discussion

Cdc48/p97 together with its cofactor Shp1/p47 is thought to work on the SNAREs in a way similar to Sec18/NSF [27,28]. Similarly, Cdc48/p97 in a complex with Ufd1 and Npl4 can act as a ubiquitin-specific chaperone. The Cdc48–Ufd1–Npl4 complex can recognise ubiquitinated proteins that are released from the ER [10–16]. Both these functions require that Cdc48 recognises 'exposed structures' on the substrate protein. Here it is shown that the ability to recognise unfolded proteins, as they appear e.g. after re-translocation from the ER, resides in Cdc48 alone and does not require cofactors. This is of interest in light of the function of ubiquitin. It has been suggested that ubiquitin changes the structure of its attached protein and thereby acts itself as a chaperone [29]. Possibly Cdc48 recognises substrates in vivo by their (ubiquitin-induced) conformation and not (only) by the presence of ubiquitin. On the other hand it is tempting to speculate that two of the evolutionary 'oldest' proteins, Cdc48 and ubiquitin, have co-evolved to serve a common function in membrane fusion and protein degradation.

Cdc48's ability to 'hold' non-native proteins seems to be independent of its ATPase function. The EM analysis of the p97 particle suggests that the major conformational change in the hexamer might occur upon ADP release rather than ATP hydrolysis [30]. Interestingly, an ATP-independent function of NSF has been found in the reassembly of Golgi membranes,

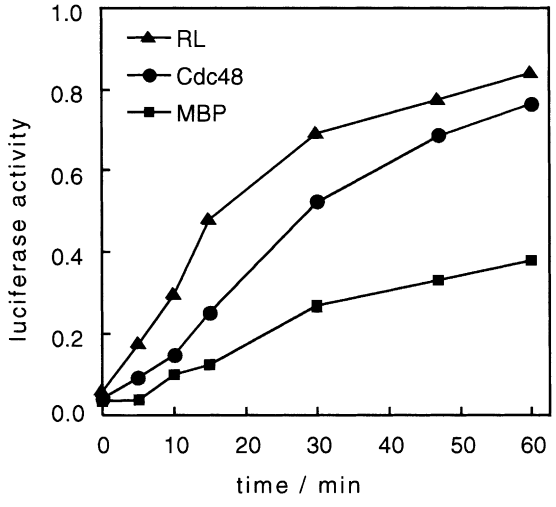

Fig. 4. Cdc48 is co-chaperone of the Hsc70–Hsp40 chaperone system. Luciferase was thermally denatured at 42°C in the presence of Hsc70 and Hsp40. To allow refolding samples were incubated at 30°C in the presence of MBP, 2.5% reticulocyte lysate or Cdc48. Luciferase activity was determined at the indicated times. Maximal luciferase was determined before denaturation and set to 1.

suggesting that NSF's ability to 'hold' SNAREs without ATP hydrolysis is sufficient for sponsoring membrane fusion [31]. Likewise Cdc48 functions without ATP hydrolysis in cell cycle progression and membrane fusion [30].

Furthermore this work shows that Cdc48 can serve as a 'holding' factor for the Hsp70–Hsp40 chaperone system, raising the possibility that Cdc48/p97 and Hsp70 systems functionally interact in vivo. In fact, Cdc48/p97 and Hsp70 (homologues) have been, independent of each other, associated with functions like membrane fusion [32], protein degradation [33], ERAD [34,35], clathrin function [36,37], and apoptosis [38]. Strikingly, Hsp104 together with Hsp70 and Hsp40, forms a chaperone system for the reactivation of heat-damaged proteins [26]. A similar system exists in bacteria, where the Hsp104 homologue ClpB ATPase cooperates with the Hsp70- and Hsp40-like DnaK and DnaJ proteins [39]. Hsp104 and ClpB belong to the AAA+ superfamily of ATPases, which also includes the AAA proteins [40,41]. Possibly, there is a more general cooperation of Hsp70-like systems with AAA proteins in vivo.

Cdc48/p97 will be able to interact with a large number of denatured and/or ubiquitinated proteins as they are inevitably present in protein extracts and in intact cells. Interaction studies of Cdc48 are therefore of limited value as long as they are not functionally corroborated.

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