

Hsp90-Cdc37 complexes with protein kinases form cooperatively with multiple distinct interaction sites

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SUMMARY

Protein kinases are the most prominent group of Hsp90 (heat shock protein 90) clients and are recruited to the molecular chaperone by the kinase specific cochaperone Cdc37 (cell division cycle 37). The interaction between Hsp90 and nematode Cdc37 is mediated by binding of the Hsp90 middle domain to an N-terminal region of *C. elegans* Cdc37 (CeCdc37). Here we map the binding site by NMR spectroscopy and define amino acids relevant for the interaction between CeCdc37 and the middle domain of Hsp90. Apart from these distinct Cdc37/Hsp90 interfaces binding of the B-Raf protein kinase to the cochaperone is conserved between mammals and nematodes. In both cases, the C-terminal part of Cdc37 is relevant for kinase binding, whereas the N-terminal domain displaces the nucleotide from the kinase. This interaction leads to a cooperative formation of the ternary complex of Cdc37 and kinase with Hsp90. For the MAP-kinase (mitogen activated protein kinase) Erk2 (extracellular-signal regulated kinase 2) we observe that certain features of the interaction with Cdc37-Hsp90 are conserved, but the contribution of Cdc37 domains varies slightly, implying that different kinases may utilize distinct variations of this binding mode to interact with the Hsp90 chaperone machinery.

The human genome encodes up to 500 kinases, which represent one of the largest families of genes in eukaryotes (1,2). They regulate essential intracellular processes like proliferation, differentiation, development, stress response and apoptosis. All protein kinases share a conserved catalytic domain, which switches between an active and an inactive state (3,4). A large number of these protein kinases are dependent on the Hsp90 chaperone system, which modulates their maturation and prevents their degradation (5-8). So far only little structural information of the chaperone-kinase complex is available (9). To facilitate kinase processing by the Hsp90 chaperone machinery, the specific cochaperone Cdc37 is required (9-11). A well-described function of this cochaperone is to slow down the ATPase activity of Hsp90 (12). It is widely expected that Hsp90 and its partner protein Cdc37 bind to the catalytic domain of the kinase (13-16). It remains elusive how the ternary complex consisting of protein kinase, Hsp90 and Cdc37 is structurally organized and how the protein kinase is processed by the chaperone system. Recently Cdc37 was observed to compete with nucleotide binding to the kinase domain of B-Raf (17), but the role of Hsp90 in these structures remains enigmatic. While Hsp90 is essential for ligand binding in case of steroid hormone receptors the

role for B-Raf activation is rather unclear (18). B-Raf is mutated in many human cancers and is known to form complexes with Hsp90 and Cdc37 in cell free systems (19). The interaction with the kinase is also weakened *in vivo* in presence of an anticancer drug that disrupts nucleotide binding to Hsp90 (20). Given the recent evolution of Hsp90 as a potential cancer target (21-23), it is important to clarify the mechanism and role of the chaperone-kinase interaction.

The molecular chaperone Hsp90 itself is a dimeric protein consisting of three domains – an N-terminal ATP binding domain, a middle domain, which is supposed to be involved in client binding, and a C-terminal dimerization domain. (24,25). ATP binding leads to a rearrangement of the three domains from an open V-shaped conformation to a closed conformation (5,26,27). It is thought that client proteins interact with the Hsp90 scaffold to stabilize destabilized conformations (6,28) and that the conformational changes performed by Hsp90's ATP driven conformational cycle are utilized for this. This is supported by the observation that inhibitors, which block ATP turnover by Hsp90, can disrupt the interaction with kinase clients (29-31).

Despite the significant homology within eukaryotic Hsp90 systems, *Caenorhabditis elegans* (CeCdc37) and human Cdc37 (hCdc37) utilize a different primary interaction site on Hsp90 *in vitro* (32,33). While the nematode cochaperone interacts with the M-domain of Hsp90, the human homolog preferentially binds to the N-terminal domain suggesting a potential two-site interaction. With only structural information on the N-terminal binding site of Hsp90 available so far, the organization of the binding site in the *C. elegans* system is unclear to date. Here we localize the interaction site of nematode Cdc37 with Hsp90 in detail and determine the differences regarding the formation of kinase complexes.

EXPERIMENTAL PROCEDURES

Cloning, Protein Expression and Purification – Human Cdc37 (hCdc37) and Hsp90 β (hHsp90) as well as the *C. elegans* homolog proteins of Cdc37 (CeCdc37, CDC-37, W08F4.8), Hsp90 (CeHsp90, DAF-21, C47E8.5) and all variants thereof were purified as described previously (34,35). The cDNA of human Erk2 was obtained from Sino Biological (Beijing, China). The pET28b plasmid was used as expression vector with the cDNA subcloned

after the N-terminal His₆-tag. For expression, transformed BL21-CodonPlus(DE3)RIL cells were grown to an OD₆₀₀ of 0.8 at 37 °C. Protein production was induced by adding 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG). Cells were disrupted in a TS 0.75 cell disruption instrument (Constant Systems Ltd., Northants, UK). The His₆-tagged proteins were trapped on a HisTrap FF 5 ml affinity column (GE Healthcare) and eluted with buffer containing 300 mM imidazole. ResourceQ ion exchange chromatography and size exclusion chromatography on a Superdex75 or Superdex200 HiLoad column (both GE Healthcare) were subsequently performed. The quality of each purified protein was confirmed by SDS-PAGE and mass spectrometry on a Bruker UltraFlex III MALDI/TOF instrument. The stabilized kinase domain of the human B-Raf kinase was expressed, purified and stored according to previously described protocols (17,36). B-Raf and Erk2 kinases were stored in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol and all other proteins were kept in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 1 mM DTT.

Alignment and structural features

A sequence alignment of the N-terminal human (AA 1-133) and nematodal (AA 1-128) Cdc37 was performed using the ClustalW2 tool (37). A secondary structure prediction of the same domain was carried out using Jpred3 with default settings (38).

ATPase activity assays – The ATP turnover of Hsp90 alone and in presence of cochaperones was analyzed in ATPase activity assays (39). To this end an ATP regenerating system containing pyruvate kinase, phosphoenol-pyruvate, NADH and lactate dehydrogenase (Roche Applied Science) was used. Measurements were performed at 25 or 30 °C using 3 μ M Hsp90 and 10 μ M of the cochaperone in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 1 mM DTT and 5 mM MgCl₂. Reactions were started by adding 2 mM ATP and the absorbance of NADH was recorded at 340 nm. Background activities were detected after adding the Hsp90 specific inhibitor radicicol (Sigma-Aldrich, USA). The activity of Hsp90 was calculated using the following equation with $\epsilon(\text{NAD}^+) - \epsilon(\text{NADH}) = -6200 \text{ M}^{-1} \text{ cm}^{-1}$.

$$Activity = \frac{\frac{\Delta A_{340}}{\Delta t} - \left(\frac{\Delta A_{340}}{\Delta t}\right)_{Background}}{(\varepsilon(NAD^+) - \varepsilon(NADH)) \cdot c(ATPase)}$$

Fluorescence labeling – Cysteine residues of CeCdc37 or hCdc37 were labeled by adding a threefold molar excess of Alexa Fluor 488 C5-maleimide (Invitrogen) to 0.5 mg protein in a buffer containing 40 mM HEPES/KOH, pH 7.5 and 20 mM KCl. After an incubation time of 1 h at room temperature the reaction was stopped by adding 20 mM DTT and free label was separated from the labeled protein on a Superdex 75 HR column (GE Healthcare). The degree of labeling (DOL) was determined by full length mass analysis on a MALDI-TOF instrument (Bruker) and by UV/VIS-spectroscopy using the following equations:

$$A_{protein} = A_{280} - A_{max} \cdot (CF_{280})$$

$$DOL = \frac{A_{max} \cdot MW}{[protein] \cdot \varepsilon_{dye}}$$

The values $CF_{280} = 0.11$ and $\varepsilon_{dye} = 71\,000\text{ M}^{-1}\text{ cm}^{-1}$ were used according to the manufacturer. The positions of the labels on CeCdc37 were determined using ESI mass spectrometry on an Orbitrap XL (Thermo Scientific).

Mass spectrometry on labeled CeCdc37 – CeCdc37 can in principle react with the dye on three cysteine residues, Cys212, Cys311 or Cys339. Samples of labeled and unlabeled CeCdc37 were analyzed to determine the labeling sites. After protein bands were excised from SDS-PAGE gels the samples were digested with trypsin. Gel pieces were then treated as described previously (40). Peptides were extracted by washing sequentially with 50 μl 0.1% formic acid and 50 μl acetonitrile. Collected supernatants were pooled, concentrated to 20 μl and filtered through a 0.22 μm Ultrafree-MC-GV centrifuge filter (Merck Millipore, Tullagreen Carrigtwohill, Ireland). Afterwards, they were loaded onto an Acclaim PepMap RSLC C18 trap column (Thermo Scientific) at a flow rate of 5 $\mu\text{l}/\text{min}$ and separated on an PepMap RSLC C18 column (75 $\mu\text{m} \times 150\text{ mm}$, 2 μM , 100 \AA , Thermo Scientific) at a flow rate of 0.2 $\mu\text{l}/\text{min}$. A linear gradient from 5 % to 35 % buffer B (ACN with 0.1% formic acid) eluted the peptides in 60 min to a LTQ Orbitrap XL (Thermo Scientific). Full

scans and 5 dependent MS2 scans (5CID spectra) were recorded in each cycle. Peptides modified by Alexa 488-C5 maleimide or iodoacetamide were detected by MaxQuant (41) and the masses of these Alexa 488-modified peptides were verified to be absent in corresponding MS1 spectra of the unlabeled control sample. Alexa 488-modifications were detected at position Cys311 and Cys339 in a highly labeled CeCdc37 sample (~85% two labels, ~15% one label), but modifications at position 212 were not observed. No iodoacetamide-modified peptide at position Cys339 was detected in highly labeled CeCdc37, implying that this residue reacts very efficiently with Alexa 488-C5 maleimide. Cys311 appears to react slightly less efficient and Cys212 may react only rarely, positioning all attached labels in the C-terminal domain.

Analytical Ultracentrifugation – Analytical ultracentrifugation (aUC) experiments were performed in a Beckman ProteomeLab XLA ultracentrifuge equipped with a fluorescence detection system (Aviv Biomedical, Lakewood, NY) and a Ti-50 rotor (Beckman Coulter, Brea) at 20 °C and 42,000 rpm as described previously (35). 250-500 nM of the labeled protein were analyzed by adding 3 to 10 μM of its unlabeled putative binding partner and optionally 4 mM nucleotides. Most measurements were performed in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 1 mM DTT and 5 mM MgCl_2 . To support the NMR analysis also measurements in 40 mM $\text{KH}_2\text{PO}_4/\text{KOH}$, pH 7.5 were performed for Cdc37 constructs in complex with Hsp90. Measurements including the kinases were performed in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl_2 , 1 mM DTT and 1 % (v/v) glycerol. dc/dt -plots were generated with the program SedView (42,43), which subtracts scans within a certain range and averages over the differentials. Plots were fit to bi-Gaussian functions to determine the $s_{20,w}$ values and the peak amplitudes.

Crosslinking with glutardialdehyde - Crosslinking experiments with glutardialdehyde (Merck, Darmstadt, Germany) were performed with fluorescently labeled CeCdc37 and hCdc37. 250 nM labeled hCdc37 and 500 nM labeled CeCdc37 were pre-incubated alone or in presence of 2 μM B-Raf in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl_2 ,

1 mM DTT and 1 % (v/v) glycerol for 10 min at 25 °C. The crosslinking reaction was initiated by the addition of 0.2 % (v/v) glutardialdehyde. After incubation time of 4 min the reaction was stopped by adding 200 mM Tris/HCl, pH 8.0. Subsequently, samples were separated on SDS-PAGE gradient gels (Serva, Heidelberg, Germany). Fluorescence signals were visualized using a Typhoon 9200 phosphor- and fluorescence imager (GE Amersham Molecular Dynamics, Minnesota, USA) with the settings appropriate for Alexa Fluor 488. To investigate the protein composition crosslinking was performed with unlabeled proteins at higher concentrations. The presence of both proteins in the respective bands was confirmed by ESI-mass spectrometry on a LTQ Orbitrap XL instrument (Thermo Scientific). Sample preparation and instrument settings were similar to the procedure described above. For crosslink measurements 2+ ions were rejected. Analysis of the dataset was performed with the MaxQuant software.

Fluorescence spectroscopy with MANT-ADP

Fluorescence measurements with 2'-3'-O-(N-Methyl-anthraniloyl)-adenosine-5'-diphosphate (MANT-ADP) (BioLog, Bremen, Germany) were used to detect its binding and release from the kinase B-Raf in presence of Cdc37 variants as previously described by Polier *et al.* (17). The release of MANT-ADP was recorded using a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon GmbH, Bernsheim, Germany). The excitation wavelength was set to 290 nm and emission spectra were recorded from 300 to 550 nm (slit widths 5 nm) at 10 °C. All measurements were performed in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol. MANT-ADP (120 μM) was incubated together with a kinase (2 μM) and optionally Cdc37 variants (3 μM) were added. Further, MANT-ADP spectra with Cdc37 variants alone were recorded to exclude quenching effects of these constructs in absence of the kinase. To correct for induced tryptophan fluorescence at the excitation wavelength of 350 nm appropriate protein spectra were recorded and subtracted from the sample spectra using the Origin 8 software package.

Anisotropy measurements - Experiments were performed with MANT-ADP (BioLog, Bremen, Germany) using the FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon GmbH, Bernsheim, Germany). Fluorescence emission

was recorded at 446 nm with an excitation at 350 nm. Polarization filters were set to the magic angle and the instrument specific G-factor was determined to be to 0.6138. The assays were performed at 10 °C in a buffer containing 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 5 % (v/v) glycerol. A baseline was recorded for 10 μM MANT-ADP before adding 3 μM B-Raf. After obtaining a stable plateau value 5 μM of different Cdc37 constructs were added to compete with MANT-ADP upon B-Raf binding. Data analysis was carried out applying a single-exponential function.

NMR spectroscopy - NMR spectra were recorded on Bruker AV900, AV750 and AV600 spectrometers equipped with TCI cryoprobe or room temperature (AV750) probe heads (Bruker, Billerica, USA) at 25 °C unless mentioned otherwise. 2D ¹H-¹⁵N water-flip-back HSQC (Heteronuclear Single Quantum Coherence) correlation experiments were measured for two CeCdc37 fragments AA 1-128 CeCdc37 and AA 36-128 CeCdc37. The proteins were isotopically labeled with ¹³C and/or ¹⁵N by growing cells in standard M9 medium containing 2 g/l ¹³C-glucose and/or 1 g/l ¹⁵N-ammonium chloride. The labeled proteins were purified as described previously and dialyzed against the storage buffer of 40 mM KH₂PO₄/KOH pH 7.5 with 8% D₂O (v/v) added. In case of AA 36-128 CeCdc37 the His₆-tag was removed with thrombin, followed by an additional purification step on a Ni-NTA column. Unlabeled CeHsp90M was added to a 1.5 excess to each construct of CeCdc37. Spectra were processed with NMRPipe (44) and analyzed using CcpNMR Analysis (45). Assignments were obtained using standard triple resonance water-flip-back backbone assignment experiments: HNCA, HNCaCb, CbCa(co)NH, HNCO, HN(ca)CO, (H)CC(co)NH TOCSY and 3D ¹⁵N-edited NOESY (46).

RESULTS

The N-terminus of CeCdc37 is sufficient to interact with CeHsp90 - Differences between human Cdc37 (hCdc37) and *C. elegans* Cdc37 (CeCdc37) regarding their interaction sites with Hsp90 were observed in previous studies (32). The nematode cochaperone binds with parts of the N-terminus to the middle domain of Hsp90 (Hsp90M), whereas for

hCdc37 (AA 138-C) the N-terminal domain of Hsp90 (Hsp90N) is the prominent binding site. So far, structural information is only available for the human cochaperone in complex with yeast Hsp90 (33). Thus, we wanted to obtain a more detailed view on the interaction in the nematode complex. We expressed and purified the N-terminal part of CeCdc37, containing amino acids 1 to 128 and performed a binding analysis of this fragment using an established aUC competition experiment with labeled full-length CeCdc37 (*CeCdc37). A complex of *CeCdc37-Hsp90 was formed and the ability of the N-terminal fragment to disrupt the complex was investigated by adding an excess amount of AA 1-128 CeCdc37 (Figure 1A). This CeCdc37 fragment could indeed compete against the full-length CeCdc37 showing its ability to interact with Hsp90 in a similar manner as full-length CeCdc37. We further tested the functional importance of AA 1-128 CeCdc37 in an ATPase assay, as CeCdc37 was found to inhibit the ATP turnover of Hsp90 (Figure 1B). The short fragment was able to decrease the ATPase activity of CeHsp90 in a similar manner as wt CeCdc37. These results imply that the CeCdc37 fragment harbouring the first 128 amino acids contains the most important parts to bind and inhibit Hsp90. Contrarily, the corresponding fragment of human Cdc37 (AA 1-133 hCdc37) was not capable of influencing the ATP turnover of CeHsp90. Instead, here the C-terminal part (AA 133-C hCdc37) contains an inhibiting activity similar to full-length hCdc37 (Figure 1B).

The CeCdc37-Hsp90M interaction depends on a Trp and an Arg residue –

To identify residues involved in the interaction between CeCdc37 and CeHsp90 we performed NMR titrations (Figure 2A,B). To this end, the CeCdc37 fragment (AA 1-128) was isotopically labeled with ^{15}N and ^1H and ^{15}N -HSQC NMR spectra were recorded. The protein was stable in the temperature range between 5 and 37 °C with only small differential chemical shift changes observed for some NMR signals with increasing temperature (data not shown).

Backbone chemical shift assignments were obtained for 94 of 124 non-proline residues. Several regions could not be assigned due to extensive overlap present in the spectra. For several residues in the C-terminal region a second minor signal is observed, potentially resulting from proline isomerization or a minor residual state in the C-terminus. Based on the

secondary chemical shift differences of the $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ resonances the majority of AA 1-128 CeCdc37 adopts an α -helical conformation (Figure 2C). In contrast residues 9-25 appear to adopt an extended conformation while the C-terminal residues 105-128 adopt a random coil conformation. These observations are consistent with secondary structure predictions employing the program Jpred which predicted this fragment of CeCdc37 as mostly α -helical (Figure 2D).

To map the binding interface with CeHsp90M spectra were recorded for ^{15}N -labeled AA 1-128-CeCdc37 alone and in the presence of CeHsp90M at 25 °C. Upon addition of CeHsp90M many of the amide signals of AA 1-128 CeCdc37 disappear or experience severe line broadening. Moreover, a number of new signals appear at different positions in the spectrum. Unfortunately, due to the poor spectra quality in the bound state and the observed instability of CeHsp90M in NMR conditions most of the new NMR signals could not be assigned (Figure 2A). Interestingly, the regions from 1-30 appear to be largely unaffected by CeHsp90M binding, while residues 44-128 show a significant reduction in signal intensities or chemical shift changes. When comparing the two spectra it is apparent that the chemical shifts of a tryptophan side chain (Trp) and an arginine side chain (Arg) are influenced by the addition of CeHsp90M. Thus, it can be expected that these two amino acids of CeCdc37 are located in a region which is influenced by the binding event. We were able to assign the tryptophan to Trp94, but were unable to unambiguously assign the corresponding arginine side chain.

Our NMR results suggest that the N-terminal region in Cdc37 is not involved in interactions with Hsp90. To confirm this we generated a shorter construct comprising only AA 36-128 lacking the His₆-tag. Using our aUC setup we find that this fragment is sufficient to bind to Hsp90 and is also able to inhibit the activity of Hsp90 in ATPase assays (Figure 3A, B). The fact that most NMR signals in the shorter construct fully superimpose with those observed for AA 1-128 CeCdc37 further confirms that the N-terminal amino acids do not (strongly) interact or modulate the structure of the core domain of Cdc37.

*Mutation of Trp94 and Arg95 disrupts complex formation –*We wanted to identify potential residues which are critical for the interaction between CeCdc37 and CeHsp90M. Our NMR experiments brought our attention to Trp94

which is next to Arg95, making them well positioned to be involved in the binding event (Figure 2D). To resolve whether these two residues play a role during complex formation, both of them were mutated to Ala residues in AA 36-128 CeCdc37. Indeed, AA 36-128 W94A/R95A CeCdc37 could not inhibit the ATP turnover of CeHsp90, nor could it compete with wt CeCdc37 for CeHsp90 binding in the aUC (Figure 3 A, B). To distinguish whether both amino acids are necessary, single point mutants were also generated. We find that already AA 36-128 W94A CeCdc37 is severely compromised in its interaction with Hsp90 and also in its inhibitory function towards the ATPase activity of Hsp90 (Figure 3A, B). Mutating only Arg95 to alanine also results in a decreased ability to inhibit the ATPase activity of Hsp90 and in a diminished complex formation with the chaperone in the aUC (Figure 3 A, B). Next, we exchanged these two residues in wt CeCdc37 (W94A/R95A CeCdc37) and analyzed its functionality and binding ability towards CeHsp90 (Figure 3A, B). Similar to the engineered point mutants in the N-terminal fragment of CeCdc37, the mutated wt protein could not bind to CeHsp90 nor could it inhibit its ATP turnover. These results show that the tryptophan at position 94 and the arginine at position 95 are involved in the binding to Hsp90 and are important to inhibit its ATP turnover in the nematode Hsp90-Cdc37 complex.

B-Raf interacts with nematode Cdc37 in similarity to human Cdc37 – Given the considerable differences between the two Cdc37 homologs during Hsp90 binding, we next aimed at studying the kinase interaction in the human and nematode Cdc37 proteins. To this end, we utilized the solubilised kinase domain of the human B-Raf kinase, which recently was shown to form a complex with human Cdc37 by Polier *et al.* (17). We performed a crosslinking experiment to test whether B-Raf interacts with Cdc37 of both species (Figure 4A). In this experiment the cochaperone was fluorescently labeled (marked with a star). *CeCdc37 and *hCdc37 by themselves produced no dimeric or higher oligomeric crosslinking products after incubation with glutardialdehyde. Interestingly, in the presence of B-Raf-kinase the *hCdc37 band is strongly diminished and a specific band at higher molecular weight appears (Figure 4A). Using mass spectrometry, this band was found to contain both proteins - Cdc37 and the B-Raf kinase. Based on the 70 kDa size of this

crosslinking product, it is to be assumed that one *hCdc37 is trapped in complex with one B-Raf molecule. The same behaviour can be observed for *CeCdc37, implying that the stabilized kinase domain of B-Raf may also directly interact with CeCdc37.

To confirm the binding of B-Raf to CeCdc37 we performed analytical ultracentrifugation with fluorescently labeled Cdc37 (Figure 4B, C). Both *Cdc37 proteins sediment with an $s_{20,w}$ value of 2.9 ± 0.2 S. In complex with B-Raf, both labeled *Cdc37 proteins sediment with a higher sedimentation coefficient (*CeCdc37: 4.1 ± 0.4 S, *hCdc37: 4.4 ± 0.3 S), confirming that despite the differences in Hsp90 interaction, both Cdc37s are capable of interacting with B-Raf in a similar manner. Polier *et al.* had further shown that ATP can disrupt the hCdc37-kinase complex (17). To see whether this effect is also observable for the nematode cochaperone, we added ATP to the preformed *CeCdc37-B-Raf complex (Figure 4B, C). Indeed the nucleotide disrupted the interaction of B-Raf with human and *C. elegans* Cdc37 leading to a dissociation of the protein complex and sedimentation of Cdc37 as an uncomplexed monomeric protein.

The N-terminal part of CeCdc37 diminishes nucleotide binding to B-Raf - The N-terminus of human Cdc37 had been shown to release a bound nucleotide from the kinase domain of B-Raf (17). We thus tested whether CeCdc37 is also able to release the nucleotide from the kinase via its N-terminal domain. A fluorescence-based assay with MANT-ADP established by Polier *et al.* (17) was used to analyze nematode Cdc37 fragments in this respect (Figure 5A, B). We could detect a competition against MANT-ADP with full-length nematode Cdc37 in similarity to the human protein. When we deleted the first 128 AA of CeCdc37, this fragment was not able to compete off the nucleotide from B-Raf anymore (Figure 5 B), implying that this mechanism could be similar to the one reported for the hCdc37 (Figure 5A). Even when deleting only the first 46 amino acids of CeCdc37, MANT-ADP remains still bound to the kinase (Figure 5B). We next aimed at analysing the reaction of nucleotide displacement kinetically. To this end, we employed an anisotropy setup of the same assay (Figure 5C). In this experiment, an increase in anisotropy signal can be observed upon binding of B-Raf to MANT-ADP. This increase is fast and happens during the mixing period. Addition of CeCdc37 results in a

decrease of the anisotropy signal consistent with the release of nucleotide. A half-time value of $t_{1/2} = 37 \pm 3$ s can be envisioned from this reaction, implying that the displacement of the nucleotide might be rather slow. We then tested different CeCdc37 constructs, whether they can result in a similar reaction. A deletion of only 25 amino acids from the N-terminus of CeCdc37 does not lead to the drop in anisotropy values in similarity to a fragment, lacking the entire N-terminal domain (Figure 5C). A fragment, lacking only the first 14 amino acids instead could still displace the nucleotide, suggesting that important residues for this reaction reside between the amino acids 15 and 25 of CeCdc37.

B-Raf binding is mediated by the C-terminal domain of Cdc37 - As B-Raf binds to CeCdc37 and hCdc37 we wondered which part of the cochaperone is additionally required to form the binding site. Using various truncation mutants we tested, whether specific deletion constructs of Cdc37 are capable to compete for B-Raf binding with *CeCdc37 in aUC experiments (Figure 6A, B). Surprisingly, a deletion of the N-terminal part of the cochaperone ($\Delta 128$ CeCdc37) was still very efficient in competing against complex formation, implying that upon deletion of the N-terminal amino acids the binding affinity is not lost, despite the inability to displace the nucleotide (Figure 6A). The same was evident in experiments with human Cdc37 fragments (Figure 6B). Performing the same assay with N-terminal Cdc37 fragments (1-128 CeCdc37 and 1-133 hCdc37) resulted in no detectable competition against full length Cdc37 (data not shown), confirming that C-terminal parts have to contribute affinity to this binding reaction to enable the N-terminal part of CeCdc37 to exchange the nucleotide.

Hence, we examined further truncation mutants of the C-terminal region of CeCdc37 (AA 128-284 CeCdc37 and AA 284-C CeCdc37). With these fragments we could not observe competition against the full length cochaperone for B-Raf binding in aUC experiments (data not shown). This highlights that the interaction with B-Raf is mediated mostly by the C-terminal part of Cdc37 with contributions of the middle domain. We additionally tested whether the truncation mutants lacking the C-terminal domain are able to prevent binding of MANT-ADP (Figure 6C, D). This indeed was not the case, confirming that the C-terminus of Cdc37 is required for binding of Cdc37 to B-Raf, while

the N-terminus of the cochaperone is required for nucleotide release.

Ternary complexes with CeHsp90 are formed with B-Raf - Having defined the interaction between Cdc37 and B-Raf, we wanted to see if also a ternary complex with Hsp90 can be formed. We used the aUC setup with labeled Cdc37 constructs and added the organism specific Hsp90 alone and in combination with B-Raf (Figure 7A, B). *CeCdc37 and *hCdc37 interact with Hsp90 alone and with B-Raf. Importantly, in the presence of B-Raf a shift of the *hCdc37-hHsp90 complex peak from 6.3 S to 7.9 S can be observed. Likewise a shift from 6.1 S to 7.0 S can be observed for *CeCdc37-CeHsp90 under the same conditions. While all *hCdc37 had formed complexes at 7.9 S, residual free *CeCdc37 is present at the same protein concentrations. These results show a slightly weaker interaction between B-Raf and CeCdc37-CeHsp90 compared to hCdc37-hHsp90. Further, they confirm that the ternary complex can be formed in both systems, pointing to a highly conserved mechanism in respect to kinase binding in these two organisms.

The protein kinase Erk2 shares specific features of the Cdc37-B-Raf interaction. - We finally wanted to know, whether other kinases are able to interact with CeCdc37 in a manner similar to B-Raf (47). To this end we utilized the labeled *CeCdc37 in analytical ultracentrifugation experiments and supplemented the MAP kinase Erk2 (Figure 8A). Similar to B-Raf, we could observe a peak shift to higher $S_{20,w}$ values (complex peak at 4.8 ± 0.4 S), showing the potential of *CeCdc37 to interact also with Erk2. We tested the specificity of these interactions by competition experiments. After addition of hCdc37, the complex of *CeCdc37 and Erk2 is reduced, but still apparent, indicating that the efficiency of complex formation is reduced compared to the B-Raf kinase. (Figure 8A). We also included ATP in these sedimentation experiments. Indeed, the ability of Erk2 to form complexes with *CeCdc37 is markedly reduced in the presence of the nucleotide showing that this feature of the interaction is conserved between B-Raf and Erk2 (Figure 8A). We further wanted to know whether N- and C-terminal fragments of hCdc37 can compete against the *CeCdc37-Erk2 complexes (Figure 8B). Here, in contrast to previous experiments with B-Raf we do not observe significant competition with

either deletion fragment, suggesting that for this kinase, the interaction requires both, the N-terminal and C-terminal parts of hCdc37. Despite these differences in the interaction mode with Cdc37, we could still observe a ternary Hsp90-Cdc37-Erk2 complex at 7.3 ± 0.3 S compared to the binary Cdc37-Hsp90 complex, which sediments with 6.8 ± 0.3 S (Figure 8C). These observations point to a chaperone assembly, which is conserved also for Erk2, but apparently with distinct features, which appear to depend on specific characteristics of the kinase.

DISCUSSION

In this study we characterized the interaction of the molecular chaperone Hsp90 and its cofactor Cdc37 with kinases. Previous experiments suggested a second binding site between the two proteins, including the N-terminal part of CeCdc37 and the middle domain of Hsp90 (32). So far, only structural information of the N-terminal domain of yeast Hsp90 with the C-terminal domain of hCdc37 is available by crystallography and NMR spectroscopy (33,48). Analyzing the structure of the *C. elegans* proteins by NMR spectroscopy, we could identify that Trp94 and Arg95 in the N-terminal domain of nematode Cdc37 are involved in the secondary binding site (Figure 9). Mutation of these sites to alanine results in loss of the interaction. In the human protein only the Trp residue is conserved, while the arginine and several other amino acids in this region are distinct. Apparently this binding site becomes undetectable if isolated from the rest of the binding interfaces in the human complex due to these amino acid changes.

Regarding the interaction between Cdc37 and protein kinases, the conserved nature between human and nematode systems is very obvious. Formation of the kinase-Cdc37 complex is readily visible in analytical ultracentrifugation experiments, crosslinking experiments and fluorescence assays. The competition between Cdc37-binding and nucleotide binding to the kinase is dependent on the presence of the N-terminal amino acids of Cdc37. Here apparently important amino acids reside in a highly conserved stretch between AA 15 and 25 and only in the presence of this part, a release of the nucleotide from the kinase can be observed. Surprisingly, we find that the interaction

between B-Raf-kinase and both Cdc37s requires the C-terminal domain of Cdc37. This allows to differentiate between a functional N-terminal part of Cdc37, harboring the nucleotide exchange activity for kinases and a C-terminal part relevant for providing the binding affinity. Further there appear to be significant variations in the interaction between Cdc37 and different kinase clients. While a similar relationship between Cdc37-binding and nucleotide binding can be observed for the kinase Erk2, the complex formation between Cdc37 and this kinase appears weaker compared to B-Raf. Interestingly, a cooperative contribution of the N- and C-terminal regions of Cdc37 could be observed for this kinase, as deletion of each region leads to a loss of interaction with the cochaperone. Even though BLAST searches show that the kinase domains of Erk2 and B-Raf share only an identity of 24%, the overall structure of these domains is very similar (KD Erk2: PDBe 3sa0; KD B-Raf: PDBe 3omv). A reason for the different binding behavior towards Cdc37 could also be the extended C-terminal tail of the Erk2 full-length protein or the fact that the B-Raf studied herein contained solely the solubilized kinase domain of the protein. These results imply that the parameters of kinase-Cdc37 complex formation can be features, which could be highly kinase dependent.

We also gained information on the binding sites used in ternary complexes. These results suggest a model, where four contacts are involved, two between Cdc37 and Hsp90 and two between Cdc37 and the protein kinase (Figure 9). The C-terminal part of Cdc37 interacts only with the kinase, whereas the N-terminal domain is bound to Hsp90 but can simultaneously interfere with the nucleotide binding pocket of the kinase. It still remains open how a client is attached to Hsp90, in particular as binding sites in the N-terminal, middle and C-terminal domain of Hsp90 have been shown to be important for client interactions (9,25,49). Given the ample number of binding sites between Cdc37 and kinase and between Cdc37 and Hsp90 it certainly argues for a closely assembled and regulated protein complex participating in the maturation of protein kinases.

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Conflict of interest - The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions - Conceived and designed the experiments: JME, MJS, KR. Performed and analyzed the NMR experiment: LF, MS. Performed the mass experiment: MAD. Performed and analyzed all other experiments: JME, MJS, KR. Wrote the paper: JMS, KR, LF, MS.

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FOOTNOTES

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⁵ The abbreviations used are: aUC, analytical ultracentrifugation; IPTG, Isopropyl- β -D-thiogalactopyranosid; CeCdc37, *C. elegans* Cdc37; hCdc37, human Cdc37; CeHsp90, *C. elegans* Hsp90; hHsp90, human Hsp90; MANT-ADP, 2'/3'-O-(N-Methyl-anthraniloyl)-adenosine-5'-diphosphate

FIGURE LEGENDS

Figure 1. Characterization of AA 1-128 CeCdc37. A. The binding of AA 1-128 CeCdc37 to Hsp90 was tested in an aUC competition experiment. A complex of 500 nM *CeCdc37 (black) and 3 μ M CeHsp90 (red) was disrupted with excess of AA 1-128 CeCdc37 (10 μ M) (blue). The illustrations symbolize labeled Cdc37 (grey with yellow star) and Hsp90 (green). B. The functionality of different Cdc37 fragments was analyzed in an ATPase assay. The activity of 3 μ M CeHsp90 was measured alone and in presence of 10 μ M of different Cdc37 constructs at 30 °C. Measurements were performed in 40 mM HEPES/KOH, pH 7.5, 20 mM KCl, 1 mM DTT, 5 mM MgCl₂.

Figure 2. NMR titration of AA 1-128 CeCdc37 with CeHsp90M. A. ¹H, ¹⁵N HSQC spectra of AA 1-128 CeCdc37 free (black) and bound with CeHsp90M (red). Inserts are 1D traces of individual peaks throughout titration of G56 bound and free of AA 1-128 CeCdc37 with CeHsp90M at 0 (black), 0.1 (dark orange), 0.2 (orange), 0.5 (orange-red), 1 (red) molar equivalents. B. Relative intensity of each peak comparing CeHsp90M bound vs. free AA 1-128 CeCdc37. C. Secondary chemical shift differences of ¹³C ^{α} and ¹³C ^{β} compared to random coil shifts. Positive and negative values indicate α -helical and β -strand conformations, respectively, values close to 0 are found for random coil conformation. Dashed line denotes regions without backbone assignments. D. Sequence alignment of AA 1-128 CeCdc37 and AA 1-133 hCdc37 using the ClustalW2 software. The stars indicate conserved AA, two dots symbolize groups of strongly similar properties and one dot represents groups of weakly similar properties. Additionally a sequence prediction was performed using Jpred3. Helical motifs are highlighted in orange and extended regions in blue. The red circle highlights the two AA relevant for the interaction with CeHsp90M.

Figure 3. Trp94 and Arg95 of CeCdc37 are crucial for binding to Hsp90M. A. The functionality of AA 36-128 CeCdc37 was tested in an ATPase assay measured at 25 °C. The activity of 3 μ M CeHsp90 was analyzed in presence of 10 μ M CeCdc37, W94A/R95A CeCdc37, AA 36-128 CeCdc37, AA 36-128 W94A CeCdc37, AA 36-128 R95A CeCdc37 and AA 36-128 W94A/R95A CeCdc37. B. An aUC competition experiment was performed with 500 nM *CeCdc37 (black) in complex with 3 μ M CeHsp90 (red), which was challenged by either 10 μ M unlabeled CeCdc37 (blue), W94AR95A CeCdc37 (orange), AA 36-128 CeCdc37 (magenta), AA 36-128 W94A CeCdc37 (green), AA 36-128 R95A CeCdc37 (marine blue) or AA 36-128 W94AR95A CeCdc37 (purple). The illustrations symbolize labeled Cdc37 (grey with a yellow star) and Hsp90 (green).

Figure 4. Cdc37-B-Raf complex formation is conserved. A. A Crosslinking experiment of labeled Cdc37 with B-Raf was performed using glutardialdehyde. 250 nM *hCdc37 is shown in lane 1 and as a crosslink with 2 μ M B-Raf in lane 3. The same experiment, employing 500nM of the *C. elegans* cochaperone, is shown in lane 2 and in complex with B-Raf in lane 4. B. An aUC experiment was performed with 500 nM labeled *hCdc37 (black) in complex with 3 μ M B-Raf (red). This complex formation was disrupted by adding 4 mM ATP (blue). In Figure C a similar aUC experiment was performed, but instead of human Cdc37 the labeled *C. elegans* protein (500 nM) was used. All measurements were carried out in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol. The illustrations symbolize labeled Cdc37 (grey with a yellow star) and the kinase (orange).

Figure 5. The very N-terminus of Cdc37 weakens binding of ADP to B-Raf. A-B. A fluorescence based assay with 120 μ M MANT-ADP (black) was performed to analyze the binding ability of 2 μ M B-Raf kinase (red). The excitation wavelength was set to 290 nm. A. The binding of the nucleotide was challenged by addition of 3 μ M hCdc37 (blue) or 3 μ M AA 133-C hCdc37 (green). B. 3 μ M CeCdc37 (blue), AA 128-C CeCdc37 (green) or AA 46-C CeCdc37 (purple) were added to the preformed MANT-ADP-B-Raf mixture. C. In addition to the fluorescence based MANT-ADP assay an anisotropy assay was performed with an excitation wavelength of 350 nm and an emission of 446 nm. After measuring a baseline of 10 μ M MANT-ADP alone, 3 μ M B-Raf was added to the assay, leading to an increase of the signal (marked with an arrow). After reaching a plateau value, 5 μ M of different CeCdc37 constructs were added: CeCdc37 (red), AA 1-284 CeCdc37 (blue), AA 15-C

CeCdc37 (purple) and AA 26-C CeCdc37 (green). All measurements were performed at 10 °C in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol (A-B) or 5 % (v/v) glycerol (C).

Figure 6. The C-terminal part of Cdc37 is required for B-Raf binding. A. An aUC experiment was performed with 250 nM labeled *CeCdc37 (black) in complex with 3 μM B-Raf (red). This complex was challenged by adding 10 μM AA 128-C CeCdc37 (blue). B. The same experiment as in A was carried out, but this time labeled *hCdc37 was used and the complex was challenged by adding 10 μM AA 133-C hCdc37. The illustrations symbolize labeled Cdc37 (grey with a yellow star) and Hsp90 (green). C. The binding ability of 2 μM B-Raf (red) towards 120 μM MANT-ADP (black) was analyzed in a fluorescence based assay. The excitation wavelength was set to 290 nm. The binding of the nucleotide was challenged by adding 3 μM AA 1-128 CeCdc37 (blue). D. The same experiment as in C was performed but the B-Raf-MANT-ADP complex was challenged with 3 μM AA 1-133 hCdc37. 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol buffer was used for all measurements.

Figure 7. Formation of the ternary complex of Hsp90-Cdc37-B-Raf. A. The formation of a ternary complex of Hsp90, Cdc37 and B-Raf was analyzed in the aUC. 250 nM *hCdc37 (black) were measured in complex with 3 μM hHsp90 (blue), 3 μM B-Raf (red) and in complex with hHsp90-B-Raf (green). B. The same aUC experiment as in A was performed but this time with 250 nM *CeCdc37 (black) in complex with 3 μM CeHsp90 (blue), 3 μM B-Raf (red) and CeHsp90-B-Raf (green). All measurements were performed in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol. The illustrations symbolize labeled Cdc37 (grey with a yellow star), Hsp90 (green) and the kinase (orange).

Figure 8. The MAP-kinase Erk2 also forms ternary complexes with Hsp90-Cdc37. A. The binding ability of Erk2 was analyzed in the aUC using 250 nM *CeCdc37 (black). The complex with 4 μM Erk2 is depicted in red and a competition experiment with 10 μM hCdc37 in blue and with 4 mM ATP in green. B. The complex of 250 nM *CeCdc37 (black) and 3 μM Erk2 (red) was also challenged with 10 μM AA 1-133 hCdc37 (blue) and 10 μM AA 133-C hCdc37 (green) in the aUC. C. 250 nM *CeCdc37 (black) were analyzed in complex with 3 μM CeHsp90 (blue) and 5 μM Erk2 (red) and in a ternary complex with CeHsp90 (3 μM) and Erk2 (5 μM) (green) using aUC. All measurements were performed in 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT and 1 % (v/v) glycerol. The illustrations symbolize labeled Cdc37 (grey with a yellow star), Hsp90 (green) and the kinase (orange).

Figure 9. Model of the ternary complex of kinase, Cdc37 and Hsp90. The model is based on the interaction sites defined for the human protein by Polier *et al.*(17) and for the nematode protein defined in this study. The interaction of kinase with Hsp90 is hypothetical, as there is only limited information, whether one domain participates or whether both subunits of Hsp90 are involved. Hsp90 is depicted in green, the kinase in orange and Cdc37 in grey. The red stars mark Trp94 and Arg95 which are relevant for the interaction of Cdc37 with the middle domain of Hsp90.

Figure 1

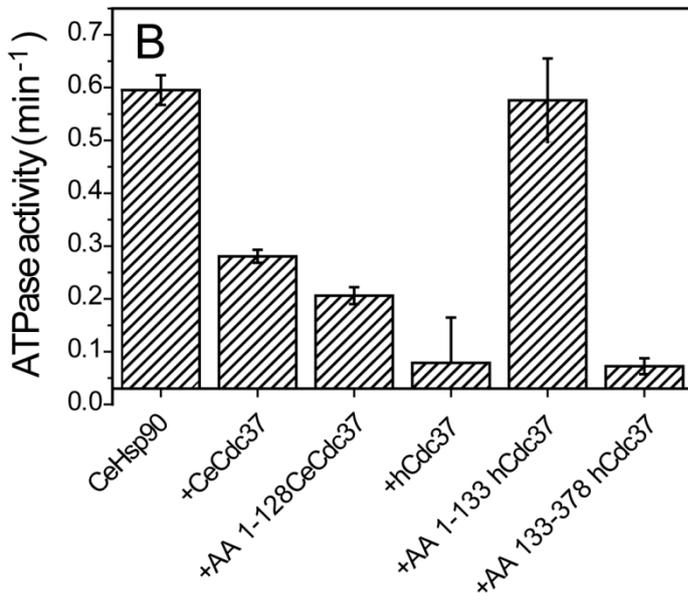
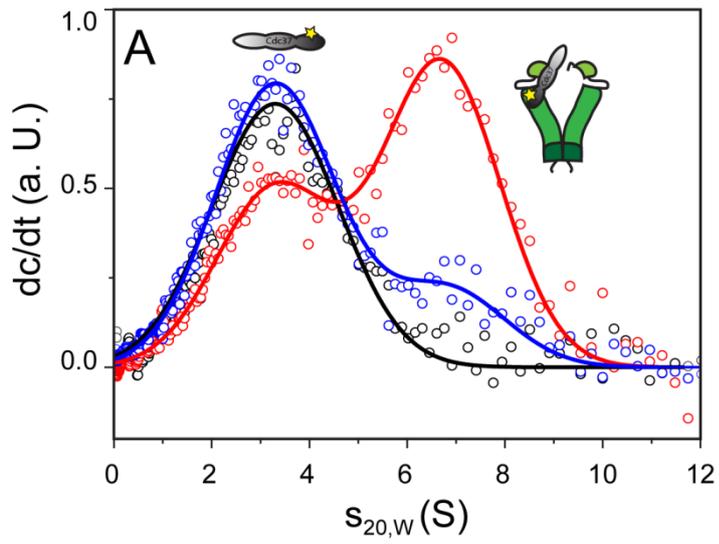


Figure 3

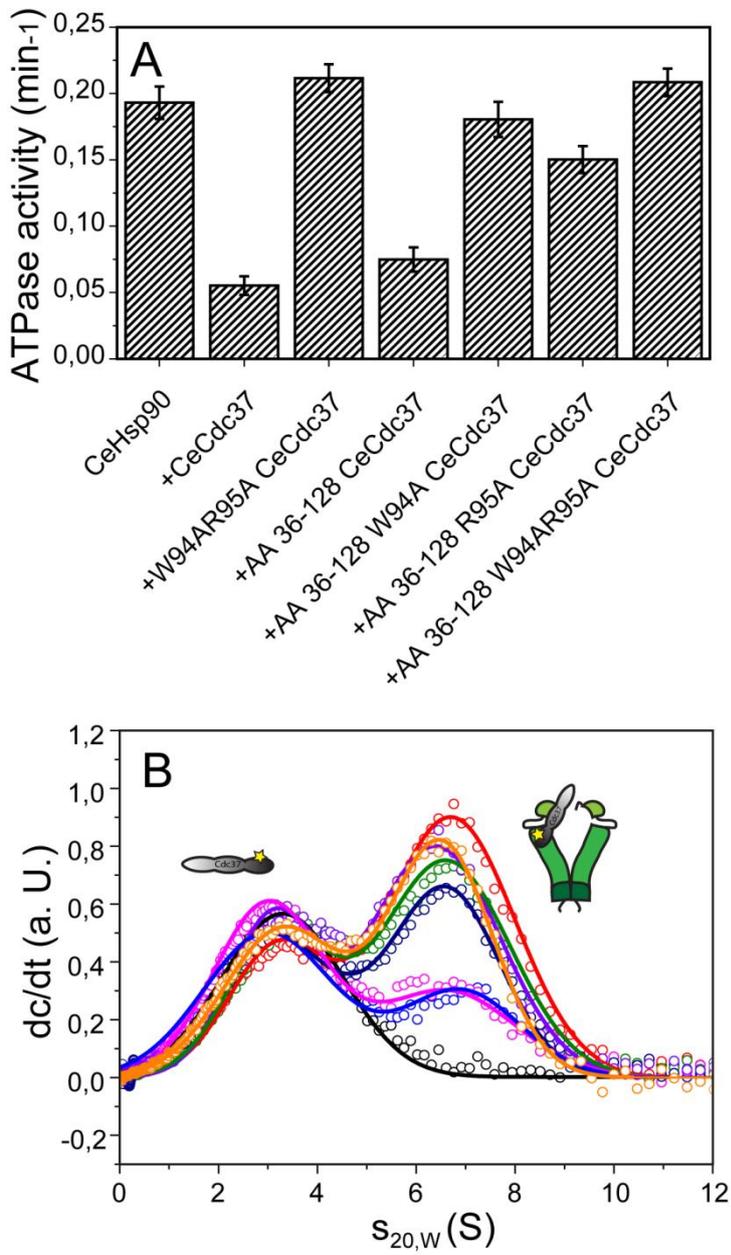


Figure 4

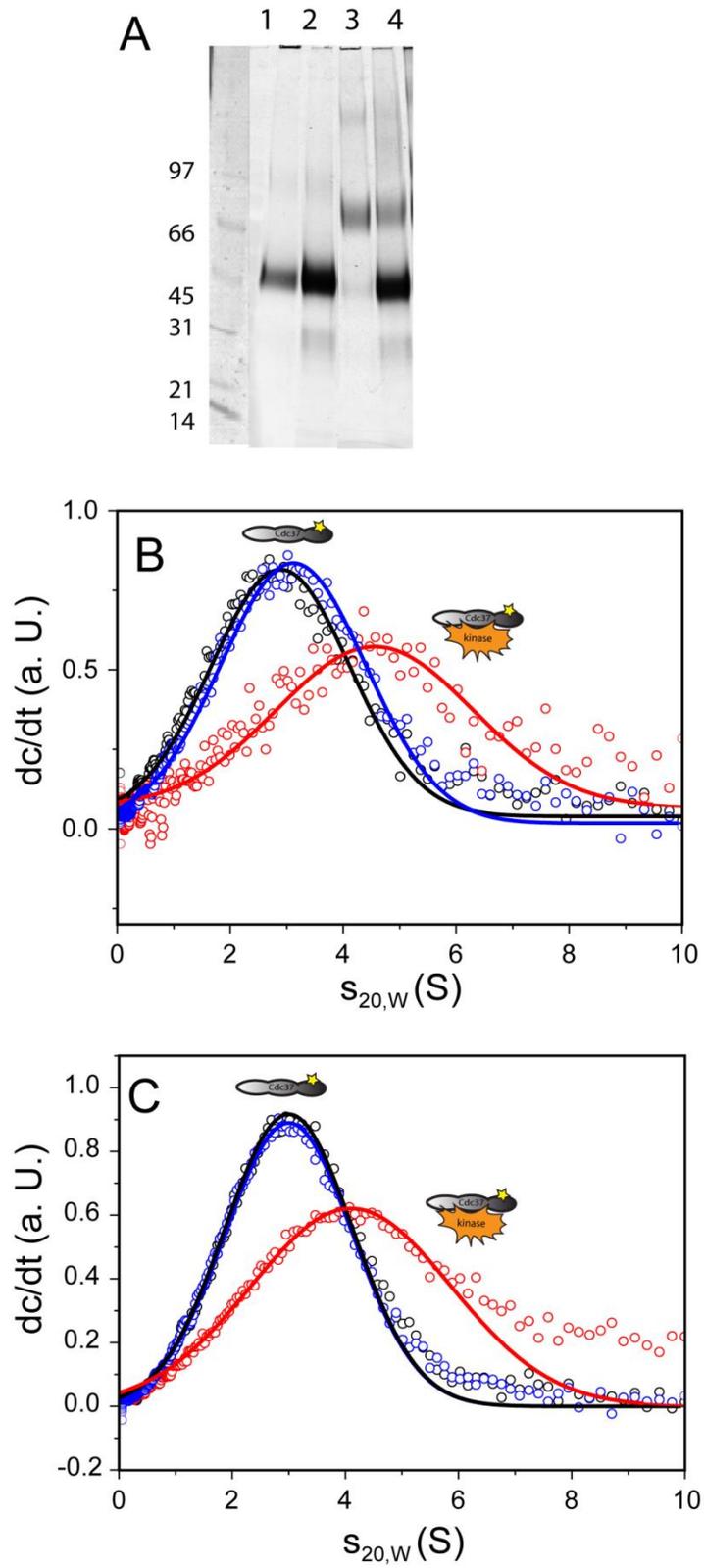


Figure 5

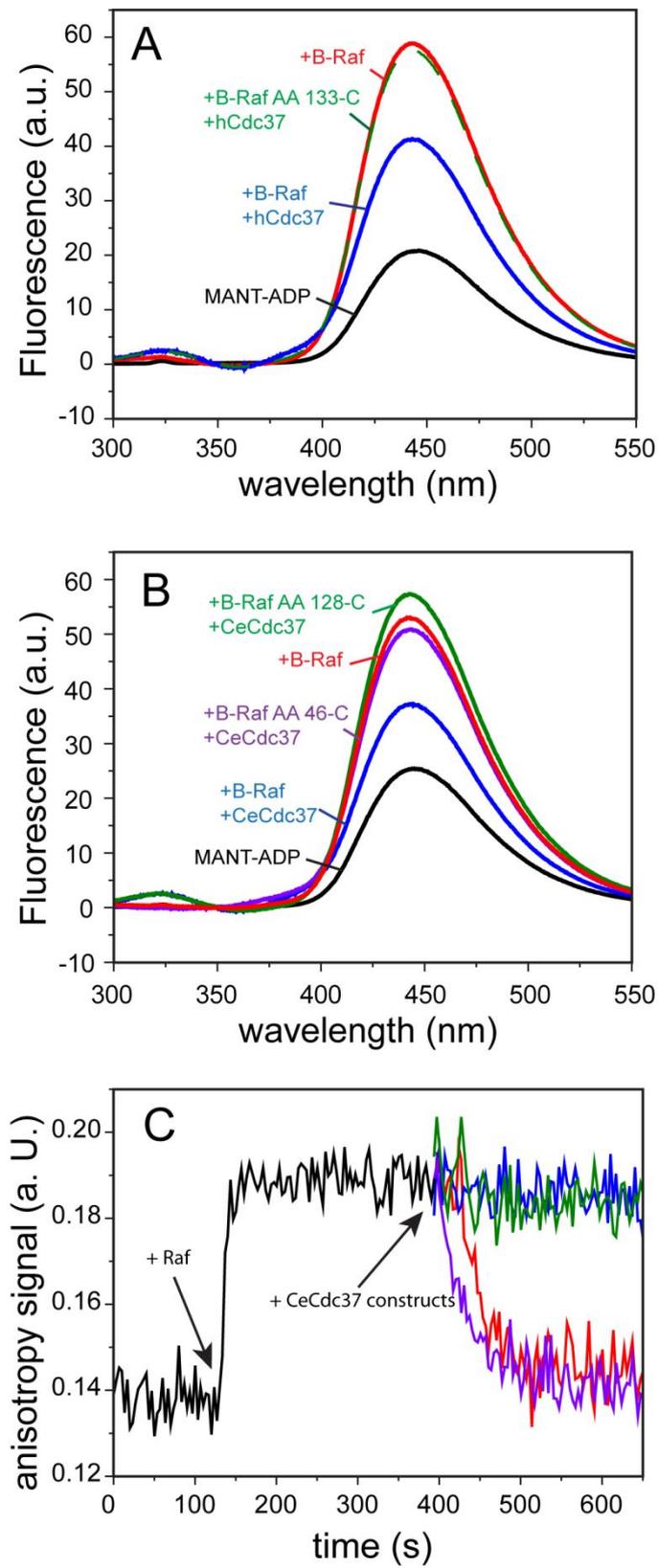


Figure 6

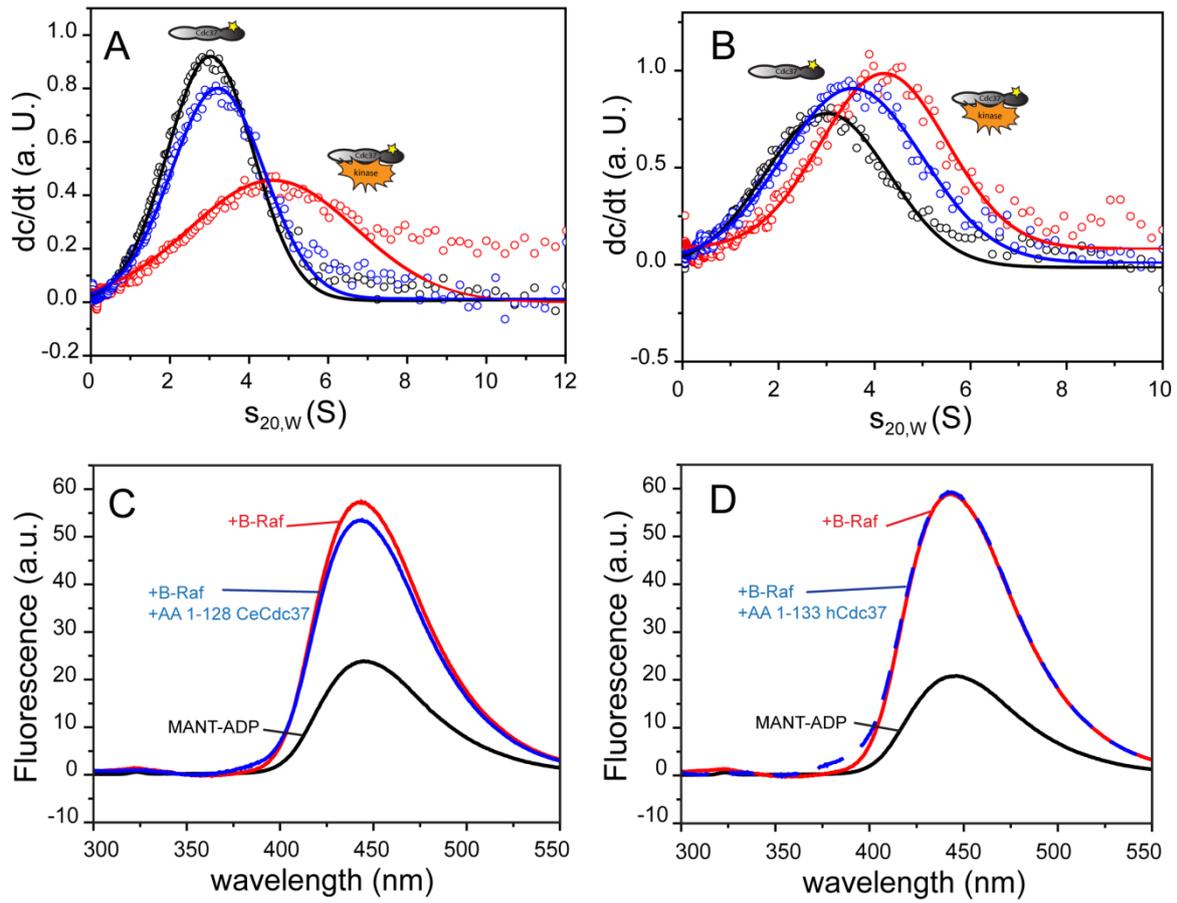


Figure 7

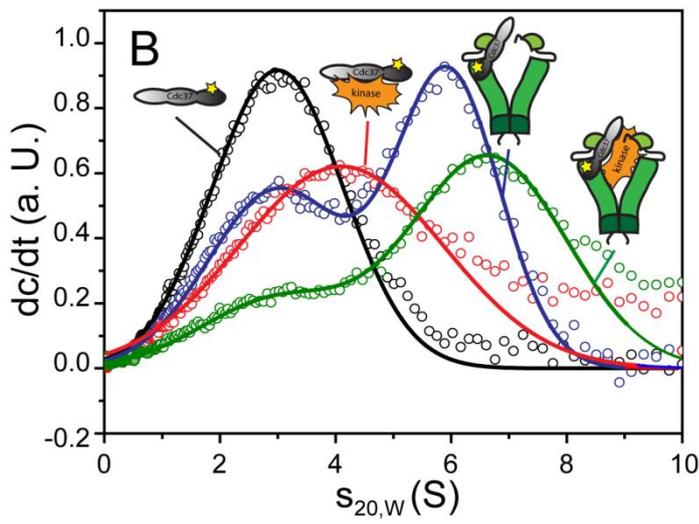
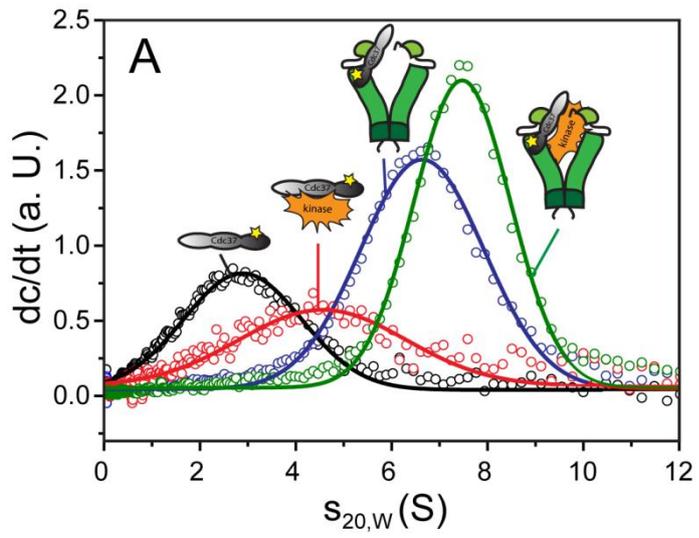


Figure 8

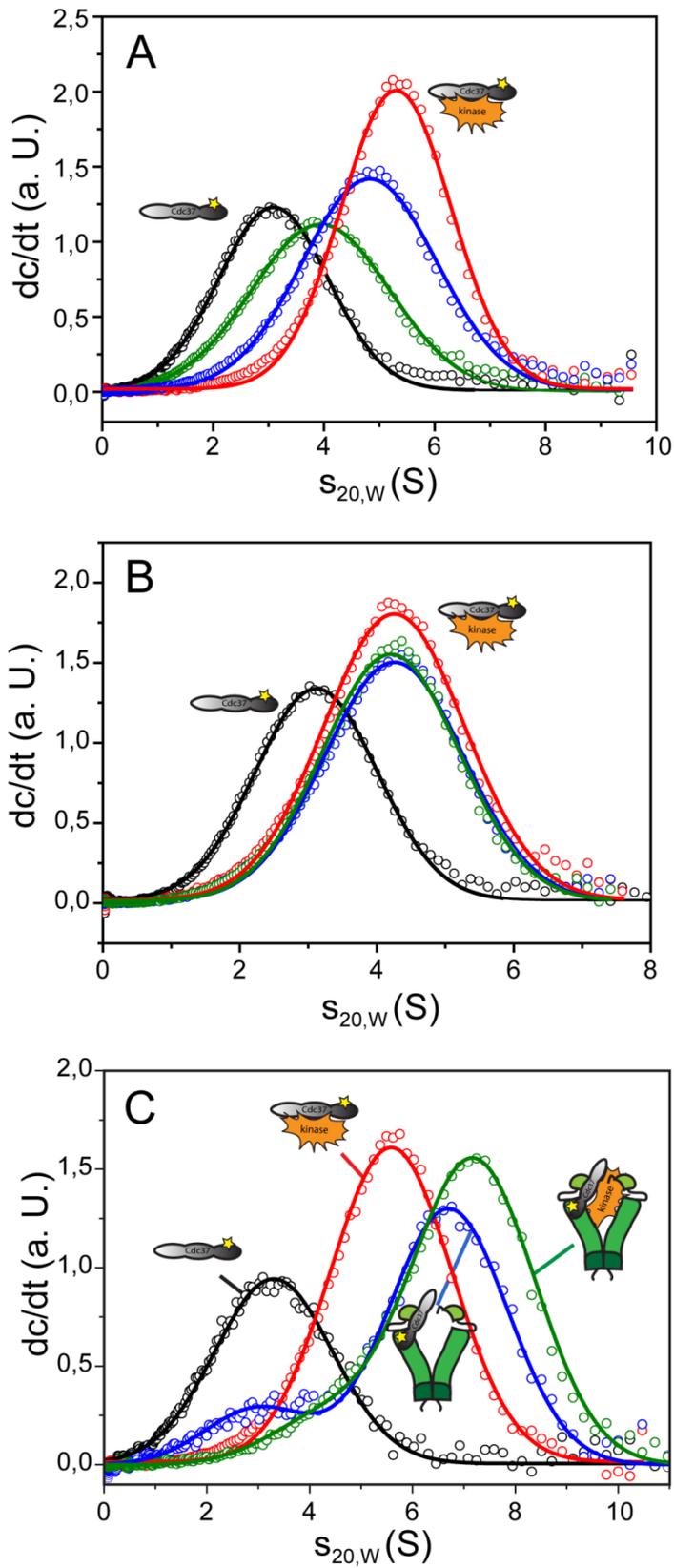


Figure 9

