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Evidence for chaperone heterocomplexes containing both Hsp90 and VCP

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Abstract

With assistance from co-chaperone partner proteins, Hsp90 plays an essential positive role in supporting the structure and function of numerous client proteins in vivo. Hsp90's co-chaperone partnerships are believed to regulate and/or target its function. Here we describe associations between Hsp90 chaperone machinery and another chaperone, the 97-kDa valosin-containing protein VCP. Coimmunoadsorption assays indicate that VCP occurs in one or more native heterocomplexes containing Hsp90 and the Hsp90 partner proteins Cdc37, FKBP52, and p23. Functional characterizations indicate that VCP is not an Hsp90 substrate, but rather demonstrate the biochemical hallmarks of an Hsp90 co-chaperone. Potential roles for a collaboration between for Hsp90 and VCP are discussed.
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The 90-kDa heat-inducible phosphoprotein (Hsp90) supports the structure and function of a diverse body of signal transducing proteins, including members from several individual kinase families, various transcription factors, and many other proteins that regulate cell growth, differentiation, and response to stimuli (for review, see [1]). Because marginally dysfunctional signal transducers need exaggerated Hsp90 support, titration of Hsp90 function unmasks cryptic mutations in cellular regulatory networks (e.g. [2–5]). Similarly, pharmacologic inhibition of Hsp90 indirectly compromises several mutant proteins that drive certain cancers (see [6]). Thus, Hsp90 is a current target in private and national efforts to develop novel compounds to treat human disease. In addition to its roles in supporting signal transducers inside the cell, recent studies suggest that Hsp90 may have other functions (e.g. [7–10]).

Although purified Hsp90 prevents protein aggregation in vitro [11–15], Hsp90 is not widely believed to function independently in vivo: instead, Hsp90 associates with a variety of partner "co-chaperones" or regulatory "cohorts" [1,16]. These partners are thought to target, regulate, and augment Hsp90's protein-binding activity. This coterie of partner proteins can be grouped into several families on the basis of their sequence similarities. One such family includes several proteins that bind to Hsp90 primarily via their tetratricopeptide repeat (TPR) domains: mammalian examples include FKBP51, FKBP52, Cyp40, p60/HOP, PP5, CHIP, XAP2, and TPR2. A second family of Hsp90 co-chaperones includes p23, a tissue-specific variant of p23 (Tsp23), and a variety of proteins containing a conserved p23-like CHORD and Sgt1 (CS) domain. A third family of Hsp90 partners is represented by Cdc37 and the related protein Harc. In addition to these three well-populated families, Hsp90 heterocomplexes can

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also include well-characterized Hsp70 and DnaJ/Hsp40 heat shock proteins, and new partnerships with Hsp90 continue to emerge [17,18]. An exhaustive list of Hsp90-associated proteins is maintained by Dr. Didier Picard and colleagues at <http://www.picard.ch/downloads/downloads.htm>.

Here, we report novel chaperone heterocomplexes containing both Hsp90 and an essential facilitator of membrane fusion and protein degradation, the AAA+ chaperone VCP (a.k.a., *Xenopus* p97 or yeast Cdc48). Consistent with this association, we also observe that VCP is found in complexes containing certain Hsp90 co-chaperones. Functional characterizations and precedents from other co-chaperone families suggest that VCP is not an Hsp90 substrate, but rather that VCP–Hsp90 complexes represent one or more novel chaperone–chaperone partnerships.

Materials and methods

Antibodies. The JJ3 monoclonal antibody against p23 was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN). The 7Dα, F5, EC1, and 34A monoclonal antibodies recognizing Hsp90, p60/HOP, FKBP52, and p48/HIP, respectively, were provided by Dr. David Smith (Mayo Clinic Scottsdale, Arizona). The C1 monoclonal antibody against Cdc37 was purchased from Affinity BioReagents (catalog MA3-029). The N27 monoclonal antibody against Hsp70 was purchased from Stressgen (catalog SPA-820). Mouse monoclonal antibody to VCP (clone 58.13.3) was purchased from Research Diagnostics (catalog PRO65278). Irrelevant control antibodies (MOPC-21) were purchased from Sigma (M7894). Polyclonal mouse antibodies to Hsp90 and Cdc37 were produced as previously described [19,20].

Preparation of cell lysates. For expression of affinity-tagged VCP, standard recombinant DNA techniques were used to insert a full-length VCP cDNA into pcDNA3.1 (InVitrogen), and sequences encoding a C-terminal hexa-histidine tag were placed in-frame with VCP coding sequences. This construction generated the recombinant VCP sequence $M_1 \ldots D_{803}L_{804}Y_{805}G_{806}$*EFHHHHHH**, wherein italics font indicates the recombinant affinity tag. For expression of tagged VCP, K562 cells were grown in RPMI 1640/FBS media and were transfected with using DMRIE-C following the manufacturer's instructions (InVitrogen).

Cell cultures were washed once in Hanks' balanced salts containing 1 mM sodium fluoride and 10 mM sodium molybdate, and were then lysed in 0.5% Igepal, 20 mM Hepes, pH 7.4, 100 NaCl, 2 mM EGTA, 1 mM DTT, 10% glycerol, 1 mM $NaVO_4$, and mammalian protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation and protein concentrations were determined via BCA assay (Pierce) and by visual inspection of PVDF membranes stained with Coomassie R-250.

Lysates of rabbit reticulocytes were prepared and supplemented with an ATP-regenerating system as previously described [21]. For in vitro translations, nuclease-treated rabbit reticulocyte lysate (RRL) was purchased from Promega, and reactions were assembled following the manufacturer's protocols.

Analysis of protein heterocomplexes. Immunoadsorptions were performed as previously described [22]. Briefly, immunoresins were prepared, mixed with either 50 μl (RRL) or 200 μl (K562) of cell lysate, and stirred on ice for 2 h. Immunocomplexes were washed with buffer containing 10 mM Pipes (pH 7.0), 0.5% Tween, and the indicated concentrations of NaCl and/or sodium molybdate. Washed immunoresins were analyzed by SDS–PAGE and Western blotting, as previously described [22].

Results

In our ongoing characterizations of Hsp90 structure and function, we have frequently observed that heterocomplexes containing p23 or Cdc37 also include an abundant protein that migrates more slowly than Hsp90 on one-dimensional electrophoresis gels (e.g., Fig. 1). The abundance of this protein in co-adsorptions varied widely among individual preparations of reticulocyte lysates, consistent with the heterogeneous nature of reticulocytes in vivo, and with the developmental regulation of chaperone levels therein [23]. The electrophoretic mobility of this protein was deceptive: it migrated very closely with Hsp90 isoforms, and might also have been predicted to similarly co-migrate with the 90-kDa isoforms of the progesterone receptor. As such, we have frequently mistaken this band for Hsp90, and we postulate that other investigators may have similarly dismissed it.

However, a close comparison of Coomassie-stained and immunostained membranes demonstrated that polyclonal anti-Hsp90 antibodies did not recognize this protein (not shown). Thus, this protein was excised from gels and subjected to trypsinolytic peptide mass fingerprinting. Mass spectrometry and database searching (not shown) convincingly identified the predominant protein in the 97-kDa band as mammalian valosin-containing protein (VCP), a.k.a. p97 in *Xenopus* oocytes and Cdc48 in yeast.

To confirm this conclusion, we isolated Cdc37 heterocomplexes by immunoadsorption and probed these complexes for the presence of VCP. When polyclonal antibodies raised against full-length Cdc37 were used

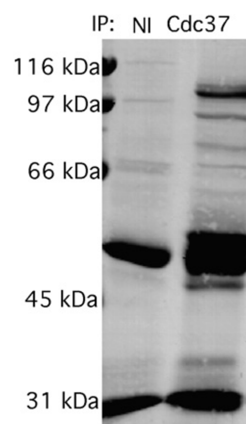

Fig. 1. Coomassie staining of Cdc37 immunoadsorptions from rabbit reticulocyte lysates. Lysates were prepared with an ATP-regenerating system prior to immunoadsorption with polyclonal mouse antibodies directed against Cdc37 (Cdc37) or with irrelevant non-immune antibodies (NI). Immunoadorptions were washed once with buffer containing 50 mM NaCl and thrice with buffer containing 20 mM NaCl. Retained proteins and size standards were resolved by SDS–PAGE and stained with Coomassie R250.

to immunoadsorb endogenous Cdc37 heterocomplexes from lysates of rabbit reticulocytes and K562 cells, subsequent Western blotting of these immunoadsorptions with anti-VCP antibodies readily detected a single protein with an M_r of 97,000 (Fig. 2A). The co-adsorption of this protein was immunospecific: although some nonspecific binding was typically observed, irrelevant control antibodies co-adsorbed only trace amounts of VCP relative to levels detected in anti-Cdc37 immunoadsorptions. In addition to rabbit reticulocyte lysates and lysates of K562 cells, VCP was similarly readily detectable in polyclonal immunoadsorptions of Cdc37 from lysates of cultured Jurkat T-cells (not shown).

To confirm the specificity of VCP's occurrence in anti-Cdc37 immunoadsorptions, anti-Cdc37 immunoadsorptions were repeated using monoclonal anti-Cdc37 antibody. Again, VCP was immuno-specifically recovered in Cdc37 heterocomplexes (Fig. 2B).

To extend and validate this observation, we also performed the reciprocal analysis: immunoadsorptions targeting VCP were probed for the co-adsorption of Cdc37. However, the VCP antibodies used in these studies did not efficiently immunoadsorb VCP. Thus, cells were transfected with plasmid DNA encoding affinity-tagged VCP (VCP-H_6). Subsequent immunoadsorptions with antibodies directed against the polyHis affinity tag of the recombinant VCP efficiently immunoadsorbed their VCP target (not shown). In addition to recombinant VCP, Cdc37 was recovered in these immunoadsorptions directed against recombinant VCP (Fig. 2C). The specificity of this adsorption was validated by identical immunoadsorptions of cells transfected with the empty expression vector. Thus, reciprocal co-immunoadsorptions demonstrated that mammalian and human cell lysates contained heterocomplexes comprised of both VCP and the Hsp90 co-chaperone Cdc37.

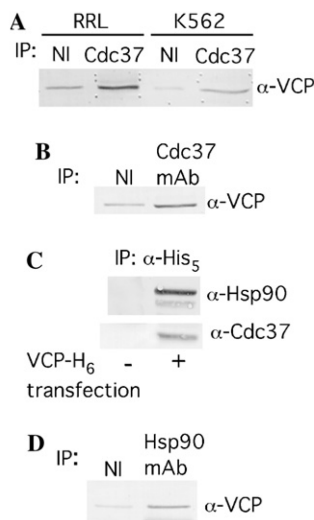

Fig. 2. Immunodetection of VCP associated with Cdc37 and Hsp90 in cell lysates. The indicated antibodies were used to immunoadsorb protein heterocomplexes from cell lysates, immunoadsorptions were washed once with buffer containing 50 mM NaCl and thrice with buffer containing 20 mM NaCl, and retained proteins were analyzed by SDS–PAGE and Western blotting. (A) Lysates of rabbit reticulocytes or of K562 cells were immunoadsorbed with polyclonal mouse antibodies directed against Cdc37 (Cdc37) or with irrelevant non-immune antibodies (NI), and immunoadsorptions were Western blotted with anti-VCP antibodies. (B) Lysates of rabbit reticulocytes were immunoadsorbed with monoclonal mouse antibodies directed against Cdc37 (Cdc37 mAb) or with irrelevant non-immune antibodies (NI), and immunoadsorptions were Western blotted with anti-VCP antibodies. (C) K562 cells were transfected with plasmid encoding affinity-tagged VCP (+ VCP-H_6) or with empty expression vector (− VCP-H_6) and cultured for 36 h. After culture, lysates were prepared and immunoadsorbed with antibodies directed against the affinity tag (α-His_5). Immunoadsorptions were Western blotted with anti-Cdc37 and anti-Hsp90 antibodies. (D) Rabbit reticulocytes were immunoadsorbed with monoclonal anti-Hsp90, and immunoadsorptions were Western blotted with anti-VCP antibodies.

To determine if Hsp90 was also a component of VCP heterocomplexes, VCP immunoadsorptions were probed for the presence of Hsp90. Like Cdc37, Hsp90 was readily detected in affinity-tagged VCP heterocomplexes, and its detection therein was specific to lysates prepared from cells expressing affinity-tagged VCP (Fig. 2C). To confirm the interaction of VCP with Hsp90, we also performed the reciprocal co-adsorption assay: monoclonal antibodies directed against Hsp90 were used to immunoadsorb Hsp90 heterocomplexes from lysates of rabbit reticulocytes. In these assays, VCP was recovered via its specific association with Hsp90 (Fig. 2D). Thus, VCP associates with Hsp90 and with the Hsp90 co-chaperone Cdc37 in lysates of rabbit and human erythrocyte progenitors.

We extended these characterizations to determine if other Hsp90 co-chaperones were present in VCP heterocomplexes. For these assays, heterocomplexes containing Hsp90, Cdc37, p23, FKBP52, Hsp70, HOP, and p48/HIP were immunoadsorbed from rabbit reticulocyte lysates. VCP was specifically detected in heterocomplexes containing Hsp90, Cdc37, p23, and FKBP52 (Fig. 3). In contrast, significant amounts of VCP did not appear to be associated with heterocomplexes containing Hsp70 or its partners HOP and p48/HIP (Fig. 3). Thus,

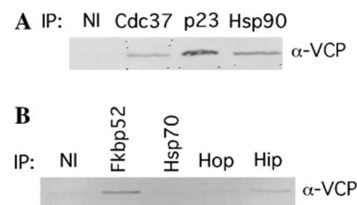

Fig. 3. Other Hsp90 co-chaperones in VCP complexes. (A,B) Lysates of K562 cells were subjected to immunoadsorption with irrelevant non-immune antibodies (NI), with polyclonal antibodies against Hsp90 and Cdc37, or with monoclonal antibodies against p23, FKBP52, Hsp70, HOP, and p42/HIP. Immunoadsorptions were washed four times with buffer containing 50 mM NaCl and subjected to Western blot analysis with anti-VCP antibodies.

VCP was detected associated with "late" Hsp90 machinery [24,25], but was not readily detected in the Hsp90–HOP–Hsp70 machine or in the Hsp70-p48/HIP machine.

To test the hypothesis that VCP was an Hsp90 client protein, we examined the effects of molybdate on the association of VCP with Hsp90 and p23. Molybdate has long been used to stabilize heterocomplexes between Hsp90 and steroid receptors, and acts by enforcing a conformation wherein Hsp90 binds tightly to its numerous client proteins. This tight binding results in prototypical interactions wherein Hsp90's binding to its clients is resistant to moderate concentrations of salt. In contrast, Hsp90's interactions with its partner proteins are typically salt labile and cannot be stabilized by molybdate (see [21] and references therein).

When this criterion was applied to VCP heterocomplexes, VCP's association with Hsp90 (Fig. 4A) and with p23 (not shown) was found to be salt-labile. This salt-lability could not be stabilized by molybdate: p97 was not retained when Hsp90 heterocomplexes were isolated from molybdate-treated lysates and washed with high-salt buffers containing molybdate (Fig. 4A). Like the Hsp90–VCP interaction, interactions between p23 and VCP were not molybdate-responsive (not shown). Thus, functional characterizations with molybdate indicated that the biochemical association of VCP with Hsp90 heterocomplexes was more similar to an Hsp90–co-chaperone interaction than to an Hsp90 client interactions.

In direct contrast to molybdate, the Hsp90 inhibitor geldanamycin enforces an alternative Hsp90 conformation that does not bind tightly to Hsp90 client proteins (e.g. [21,26]). Thus, we treated MCF-7 cells with geldanamycin and assessed Hsp90–VCP interactions therein. These analyses indicated that prolonged incubation of MCF-7 cells in the presence of geldanamycin did not hinder the subsequent detection of VCP in Hsp90 complexes, i.e., geldanamycin did not disrupt the Hsp90–VCP interaction (Fig. 4B). This resistance to geldanamycin was wholly inconsistent with the assignment of VCP as an Hsp90 client, suggesting instead that VCP was an Hsp90 partner protein.

To confirm this interpretation, we characterized the impact of geldanamycin on VCP levels in living cells. Geldanamycin enforces a specific conformation of Hsp90 that does not support the function of Hsp90's typical client proteins, thus leading to their in vivo degradation via a variety of proteolytic pathways (e.g. [26–28]). Using this tool, VCP levels in geldanamycin-treated K562 cells were characterized by Western blotting. VCP levels remained constant throughout prolonged culture in the presence of geldanamycin (Fig. 4C). This stability was, again, wholly inconsistent with the assignment of VCP as an Hsp90 client, suggesting instead that VCP was an Hsp90 partner protein.

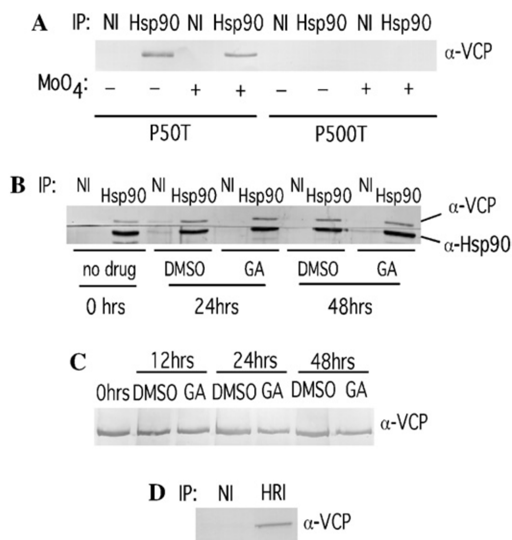

Fig. 4. Effects of geldanamycin and molybdate on VCP. (A) Rabbit reticulocyte lysates were supplemented with an ATP-regenerating system for 30 min at 30 °C, after which one-half of the reaction was treated with 20 mM sodium molybdate for 1 min. Reactions were then chilled and immunoadsorbed with irrelevant non-immune antibodies (NI) or with antibodies directed against Hsp90 (Hsp90). Immunoadsorptions were washed four times with buffer containing 50 mM NaCl (P50T). Alternatively, immunoadsorptions were washed twice with buffer containing 50 mM NaCl and twice with buffer containing 500 mM NaCl (P500T). Washed immunopellets were subjected to Western blotting with anti-VCP antibodies. (B) MCF-7 cells were cultured in the presence of 0.1 μM geldanamycin (GA) or drug vehicle (DMSO) for the indicated times, and cell lysates were immunoadsorbed with either irrelevant control antibodies (NI) or with anti-Hsp90 antibodies (Hsp90). Immunoadsorptions were washed four times with buffers containing 50 mM NaCl, and retained proteins were analyzed by Western blotting with anti-VCP and anti-Hsp90. (C) K562 cells were cultured in the continuous presence of 0.1 μM geldanamycin (GA) or with an equal volume of drug vehicle (DMSO) for the indicated times, lysed, and cell lysates were subjected to immunoblotting with antibodies directed against VCP. (D) Rabbit reticulocyte lysates were supplemented with an ATP-regenerating system and immunoadsorbed with irrelevant control antibodies (NI) or with antibodies directed against the Hsp90-dependent kinase HRI (HRI). Immunoadsorptions were washed twice with buffer containing 50 mM NaCl and twice with buffer containing 150 mM NaCl prior to analysis by SDS–PAGE and Western blotting with anti-VCP.

Because VCP did not appear to be an Hsp90 client protein, we tested the hypothesis that VCP might bind directly to an Hsp90 client. For these characterizations, antibodies directed against the Hsp90-dependent kinase HRI were used to immunoadsorb rabbit reticulocyte lysates. These adsorptions detected the specific co-adsorption of VCP within HRI heterocomplexes (Fig. 4D), indicating that VCP occurs in one or more heterocomplexes with the Hsp90 client protein HRI.

Discussion

Physical and functional characterizations presented here suggest that Hsp90 and VCP are co-chaperone part-

ners. Physically, co-immunoadsorption assays demonstrate that VCP occurs within one or more heterocomplexes containing Hsp90, and in complexes containing the Hsp90 partners Cdc37, FKBP52, and p23 (Figs. 2 and 3). These findings are highly consistent with previous observations that Hsp90 and VCP co-purify during chromatographic separations [29]. Functionally, however, our results do not support designating VCP as an Hsp90 client. Unlike prototypical Hsp90 clients, VCP associates with Hsp90 in a manner that is not responsive to the well-characterized Hsp90 inhibitors molybdate and geldanamycin (Fig. 4). Furthermore, VCP is not targeted for degradation in response to Hsp90 inhibition by geldanamycin (Fig. 4). Because VCP does not demonstrate these prototypical client responses to Hsp90 inhibition, VCP is instead implicated as an Hsp90 partner protein, i.e., a co-chaperone. This conclusion is also consistent with VCP's binding to a well-characterized Hsp90 client, namely the heme-regulated protein kinase (HRI), present in rabbit reticulocytes (Fig. 4).

As an alternative possibility, however, Hsp90 has recently been described as a substrate for ubiquitinylation [30], suggesting that the heterocomplexes we describe here represent a role for VCP in targeting ubiquitinylated Hsp90 for degradation. Currently, we disfavor this interpretation on the basis of four arguments: (i) the cells used in these studies were not subjected to prolonged drug treatments analogous to those reported to induce Hsp90 ubiquitinylation [30]; (ii) anti-Cdc37 antibodies co-adsorb VCP versus previous reports that ubiquitinylation of Hsp90 inhibits Hsp90's interactions with Cdc37 [30]; (iii) ubiquitinylation of Hsp70 does not prompt its degradation [31], suggesting that ubiquitinylation of Hsp90 and Hsp70 might instead generate new chaperone partnerships [32]. (iv) Other observations and arguments, advanced below, support the existence of an Hsp90–VCP co-chaperone partnership. Nonetheless, the possibility that VCP might bind a sub-population of ubiquitinylated Hsp90 deserves full consideration.

The postulated partnership between Hsp90 and VCP is fully consistent with previous descriptions of cooperation among molecular chaperones. In various protein folding and degradation pathways, core chaperones from the Hsp90, Hsp70, and AAA+ families must specifically recognize and bind an enormous range of substrates. To provide substrate specificities that are both "broad and high," alternative domain modules and various partner co-chaperones supplement and regulate the peptide-binding activity of the core chaperone [33]. Such coordinated and presumably multi-valent binding thus allows a finite pool of conserved machineries to specifically recognize diverse bodies of protein substrates (for discussion, see [33]). Salient examples include the well-documented Hsp90–Hsp70 partnership on the HOP machine, an interaction governed by acidic C-terminal motifs present on both Hsp90 and Hsp70 [34,35]. Similarly, Hsp90 has recently been demonstrated to occur in regulated protein heterocomplexes with a second ATP-regulated chaperone, namely Hsp104, an interaction that is mediated by C-terminal conserved acidic sequences present on each chaperone [36]. Similarly, the C-termini of both Hsp90 and VCP display two acidic motifs, one of which is highly conserved between the two chaperones: [(V/I)(D/E)-E-D-D-P]. The postulated partnership between VCP and Hsp90 is also consistent with their other partnerships. Both VCP and Hsp90 are physically associated with ubiquitin ligases [37–40], with proteins that orchestrate transport of mitochondrial proteins across membranes [41,42], and with the proteasome [29,43–45]. Thus, the possibility of a VCP–Hsp90 partnership should be added to our growing list of potential chaperone collaborations.

In addition to physical overlaps, functional overlaps between Hsp90 and VCP are implied by limited evidence that Hsp90 might orchestrate certain protein degradation pathways. While VCP chaperones ubiquitin-mediated protein degradation [46], Hsp90 is typically viewed to fulfill other functions [1]. However, many Hsp90 client proteins are ubiquitinylated as normal aspects of their molecular physiology or in response to Hsp90 inhibition [1]. Additionally, Hsp90 has been speculated to deliver substrates for degradation fates [44], and a limited number of studies provide biochemical evidence for such a role [45,47–51]. Thus, common "delivery for proteolysis" roles for VCP and Hsp90, and the physical interactions that we have described here, imply that Hsp90 and VCP may cooperate in certain degradation pathways. Similarly, Hsp70, a known Hsp90 partner, has been implicated as a proteasome co-chaperone (see [52]).

As another possibility, Hsp90–VCP–Cdc37 heterocomplexes might reflect roles for Hsp90 and Cdc37 in directing one or more tyrosine kinases to VCP. This speculation arises from the observation that VCP is a major in vivo target of tyrosine kinases, and tyrosine phosphorylation of VCP regulates its intracellular localization [53–58]. In this regard, roles for Hsp90 and Cdc37 in governing VCP phosphorylation are suggested by the observation that numerous tyrosine kinases require Hsp90 and Cdc37 for kinase support, regulation, and/or localization [1].

Summarizing, results presented here document the existence of a novel chaperone complexes containing VCP, Hsp90, and three of Hsp90's co-chaperones. These heterocomplexes are postulated to represent a novel chaperone collaboration, whose function remains to be determined.

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