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Folding and Quality Control of the VHL Tumor Suppressor Proceed through Distinct Chaperone Pathways

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Summary

The mechanisms by which molecular chaperones assist quality control of cytosolic proteins are poorly understood. Analysis of the chaperone requirements for degradation of misfolded variants of a cytosolic protein, the VHL tumor suppressor, reveals that distinct chaperone pathways mediate its folding and quality control. While both folding and degradation of VHL require Hsp70, the chaperonin TRiC is essential for folding but is dispensable for degradation. Conversely, the chaperone Hsp90 neither participates in VHL folding nor is required to maintain misfolded VHL solubility but is essential for its degradation. The cochaperone HOP/Sti1p also participates in VHL quality control and may direct the triage decision by bridging the Hsp70-Hsp90 interaction. Our finding that a distinct chaperone complex is uniquely required for quality control provides evidence for active and specific chaperone participation in triage decisions and suggests that a hierarchy of chaperone interactions can control the alternate fates of a cytosolic protein.

Introduction

Efficient clearance of nonnative polypeptides is essential for cell survival. Failure to eliminate misfolded proteins can result in toxic aggregates associated with conformational diseases such as Parkinson's and Huntington's (Horwich and Weissman, 1997; Dobson, 1999). In bacteria, ATP-dependent AAA proteases degrade misfolded proteins, while eukaryotes primarily utilize the ubiquitin-proteasome pathway to fulfill this role (Dougan et al., 2002; Goldberg, 2003). The mechanisms that enforce quality control of cytosolic proteins and the components that target a nonnative polypeptide for elimination remain ill defined despite their critical importance for cell viability.

Molecular chaperones, which bind nonnative polypeptides and promote their folding to the native state (Bukau and Horwich, 1998; Frydman, 2001; Hartl and Hayer-Hartl, 2002), are thought to aid in the destruction of misfolded proteins. For example, quality control in the secretory pathway, known as ERAD (endoplasmic reticulum-associated degradation), requires the participation of ER chaperones and the cytosolic AAA-ATPase Cdc48p (Fewell et al., 2001; Hampton, 2002; Kostova and Wolf, 2003). Likewise, chaperones participate in the degradation of folding-defective cytosolic proteins in *E. coli* and mitochondria (Dougan et al., 2002; Kandror et al., 1994; Kaser and Langer, 2000). In eukaryotes, in vitro experiments demonstrate a requirement for Hsp70 in the degradation of some misfolded proteins (Bercovich et al., 1997), while in vivo experiments in yeast implicate the Hsp70 cofactor Ydj1p in this process (Lee et al., 1996). However, these studies do not clarify the question of how chaperones contribute to the triage decision that targets polypeptides for degradation. Understanding how chaperones help select nonnative proteins for degradation has been hindered by the use of grossly misfolded or "abnormal" polypeptides as models. Given that these proteins cannot fold under any circumstance and can only either be degraded or aggregate, they are not adequate to address the mechanism of a physiological triage decision. Based on these studies, a simple model for protein quality control was proposed, whereby chaperones perform a passive "holding" function, maintaining the solubility of misfolded intermediates, thus facilitating sampling by the ubiquitination machinery (Wickner et al., 1999). In this view, the role of chaperones in quality control is an extension of their primary role in folding, in which repeated attempts to fold a defective protein, through cycles of chaperone binding and release to the bulk solution, would eventually result in passive partitioning to the degradation machinery (Wickner et al., 1999). A distinguishing feature of this model is that the requirement for chaperones in quality control stems primarily from the need to maintain the solubility of misfolded intermediates and is thus nonspecific. Consequently, any chaperone system capable of binding a nonnative polypeptide may assist its degradation. An alternative model, raised by recent observations that some chaperones specifically interact with components of the ubiquitin-proteasome pathway (Verma et al., 2000; Cyr et al., 2002), envisions that certain cellular chaperones play an active and specific role in selecting a polypeptide for cytosolic quality control (McClellan and Frydman, 2001). Accordingly, inactivation of these particular chaperones should compromise the degradation of misfolded proteins.

Here we sought to distinguish between a passive, nonspecific model of chaperone action and an active and specific role for chaperones in cytosolic quality control. To this end, we studied the chaperone requirements for the degradation of a physiologically relevant substrate of the quality-control machinery. Unlike previously studied model substrates for cytosolic quality control, the chaperone-mediated folding pathway of the von Hippel-Lindau (VHL) tumor-suppressor protein is well characterized (Feldman et al., 1999; Feldman et al., 2003; Hansen et al., 2002; Melville et al., 2003), affording us the opportunity to compare the chaperone requirements for folding and degradation. Studies in yeast and mammalian cells indicate that both Hsp70 and the chaperonin TRiC are required for correct folding of newly translated VHL, which is coupled to assembly of a ternary complex with its partner proteins elongin B and C (VHL-elongin BC, herein VBC complex; Feldman et al., 1999; Feldman et al., 2003; Hansen et

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al., 2002; Melville et al., 2003). These chaperones appear to function sequentially in the VHL folding pathway, with loss of Hsp70 function blocking association with TRiC and loss of TRiC function having no effect on Hsp70 association (Feldman et al., 2003; Melville et al., 2003). Importantly, loss of VHL function is associated with a number of inherited and spontaneous tumors (Kaelin, 2002), and several tumor-causing mutations are known to disrupt elongin BC binding and abrogate VHL folding (e.g., Feldman et al., 1999), leading to VHL degradation by the proteasome (Schoenfeld et al., 2000).

In this study we establish that the chaperone systems required for VHL degradation are different from those required for its folding. While the chaperonin TRiC/CCT is required for VHL folding, it is dispensable for degradation. In contrast, Hsp90 is not required for VHL folding but is specifically essential for its degradation. Our findings are inconsistent with proposed models for cytosolic quality control whereby the same chaperones that promote folding of a polypeptide will also facilitate its passive partitioning to the degradation machinery. Because VHL is still soluble and associated with chaperones, including Hsp70 and TRiC, in Hsp90-deficient cells where degradation is prevented, it appears that the role of chaperones in quality control is not limited to passively maintaining solubility of misfolded intermediates. Instead, it appears that only some cellular chaperones can target misfolded VHL for degradation, supporting the idea that specific, rather than stochastic, chaperone pathways act in folding and degradation. The identification of two distinct pathways of chaperone interactions for VHL, one leading to folding and one to degradation, indicates that the fate of a cytosolic protein may be controlled by a hierarchy of chaperone interactions. These findings provide novel insights into the logic underlying triage decisions in the eukaryotic cytosol.

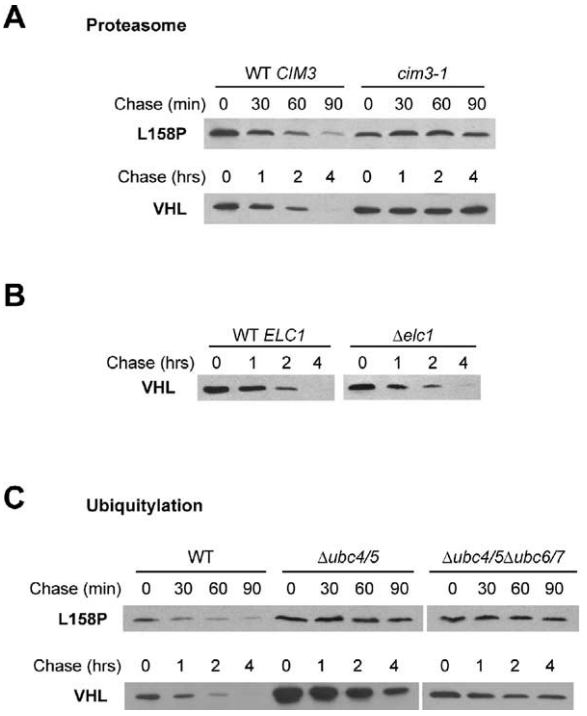

Figure 1. VHL Is Degraded by the Ubiquitin-Proteasome Pathway

(A) VHL/L158P expression in wild-type (wt *CIM3*) or proteasome mutant (*cim3-1*) yeast was repressed by the addition of glucose, and samples were collected at the indicated chase times. Data are presented as anti-VHL immunoblots of the resulting cell extracts.

(B) VHL degradation was examined in wt *ELC1* and *Δelc1* strains as described above.

(C) Immunoblot analysis of VHL/L158P degradation rates in wt yeast, isogenic strains lacking *UBC4* and *UBC5*, and yeast deleted for *UBC4*, *UBC5*, *UBC6*, and *UBC7*.

Results

Misfolded VHL Is Degraded by the Ubiquitin-Proteasome Pathway

VHL folding is coupled to its assembly into a ternary complex with its partner proteins elongin B and C. In mammalian cells, tumor-causing mutations in VHL that disrupt elongin BC binding, such as L158P, abrogate VHL folding (Feldman et al., 1999) leading to its degradation by the proteasome (Schoenfeld et al., 2000; see also Figure S1). To establish whether VHL quality control proceeds similarly in yeast, folding-defective mutant L158P VHL was expressed in *Saccharomyces cerevisiae* under the control of a galactose-regulated promoter. Glucose addition repressed expression, allowing us to follow the fate of misfolded VHL. L158P VHL was degraded with a half-life of ~40 min (Figure 1A, L158P, left; see also Figure S6B). As described (Melville et al., 2003; Schoenfeld et al., 2000), wild-type (wt) VHL expressed in the absence of elongin BC also fails to fold and is degraded, with a comparable, albeit slightly longer, half-life (~100 min; Figure 1A, VHL, left; see also Figure S6A). In principle, the observed difference in half-life of L158P and wt VHL could arise from partial stabilization of wt VHL by the yeast elongin C homolog Elc1p, which can bind in vitro to VHL-derived peptides (Botuyan et al., 1999). This is not the case, however, as deletion of *ELC1* did not alter the rate of wt VHL degradation (*Δelc1*; Figure 1B). The difference in half-life may instead arise from conformational differences between these misfolded VHL variants.

We next examined the role of the ubiquitin-proteasome pathway in VHL degradation. Both misfolded VHL variants were stabilized in the proteasome mutant strains *cim3-1* and *pre1-1* (Figure 1A, right-hand panels and data not shown). Furthermore, VHL was also stabilized by the deletion of specific ubiquitin-conjugating enzymes (UBCs or E2s, Figure 1C), indicating that VHL degradation is ubiquitin dependent. While L158P VHL was strongly stabilized in cells lacking the functionally redundant *UBC4* and *UBC5* E2s (Figure 1C), full stabilization of wt VHL required the additional deletion of the E2 pair *UBC6*/*UBC7* (Figure 1C). This discrepancy further suggests that conformational differences between these VHL variants may influence their ubiquitination pathway. Interestingly, *UBC4*/*UBC5* and *UBC6*/*UBC7* were previously implicated in the degradation of other misfolded proteins (Hampton, 2002; Seufert and Jentsch, 1990). Altogether, these data establish that in yeast, as in mammalian cells (Schoenfeld et al., 2000), misfolded

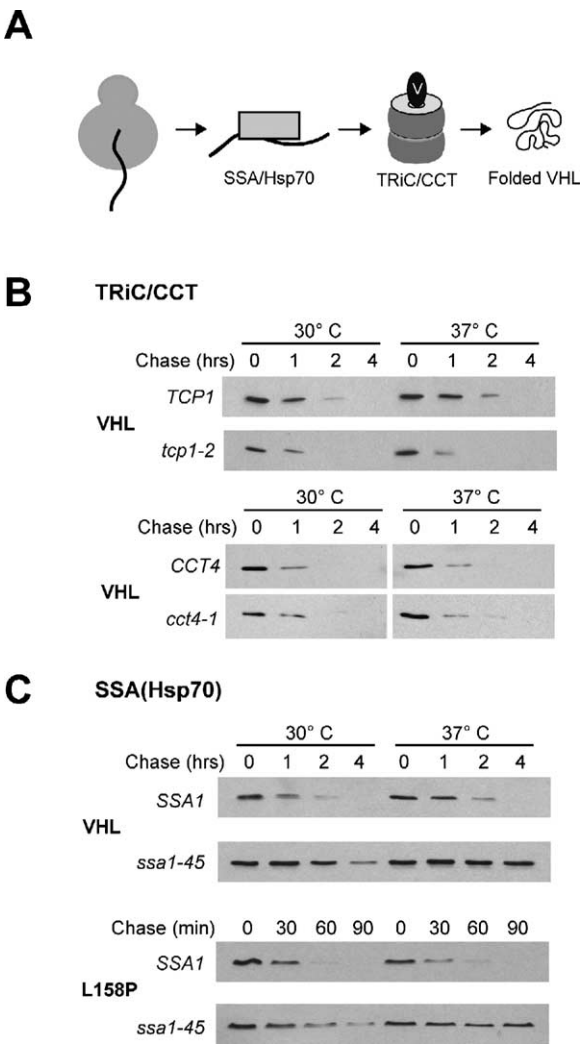

Figure 2. Role of TRiC/CCT and Hsp70 in VHL Degradation

(A) Scheme: chaperone-mediated folding of VHL requires both Hsp70 and TRiC.

(B) The steady-state degradation of VHL was assessed at 30°C and 37°C in wt yeast or yeast with a temperature-sensitive mutation (*tcp1-2; cct4-1*) in the TRiC subunits *TCP1* and *CCT4*.

(C) VHL/L158P degradation was examined in cells harboring deletions of *SSA2-4* and containing either wt *SSA1*or a temperature-sensitive allele, *ssa1-45*, at both 30°C and 37°C.

VHL degradation proceeds via the ubiquitin-proteasome system.

Degradation of Misfolded VHL Requires Specific Molecular Chaperones

Correct VHL folding in both mammalian and yeast cells is mediated by a chaperone pathway consisting of Hsp70 and the chaperonin TRiC/CCT (Figure 2A; Feldman et al., 1999; Feldman et al., 2003; Melville et al., 2003). We therefore next examined whether these chaperones, required for VHL folding, also participate in the elimination of both misfolded variants: tumor mutant L158P and wt VHL expressed in the absence of elongin BC. The role of TRiC in VHL degradation was examined in two chaperonin-defective strains harboring temperature-sensitive alleles in TRiC subunits 1 (*tcp1-2*; Ursic et al., 1994) and 4 (*cct4-1/anc2-1*; Vinh and Drubin, 1994). Incubation at the nonpermissive temperature, which abolishes VHL folding (Melville et al., 2003), did not prevent VHL degradation in either strain (37°C; Figure 2B; see also Figure S6Ai). Thus, TRiC function, while required for VHL folding, is dispensable for the degradation of misfolded VHL.

Next, the role of Hsp70 was assessed using the *ssa1-45* temperature-sensitive strain and the isogenic strain containing wt *SSA1* (Becker et al., 1996; Melville et al., 2003). Whereas VHL degradation in *SSA1* cells was unaffected by the shift from 30°C to 37°C (Figure 2C, top panel), L158P and wt VHL were strongly stabilized in *ssa1-45* cells at the nonpermissive temperature (37°C; Figure 2C; see also Figures S6Aii and S5Bi). Importantly, the ubiquitin-proteasome system is fully functional in the absence of *SSA* function, as the incubation of *ssa1-45* cells at the nonpermissive temperature does not compromise the proteasome-dependent degradation of other substrates (Brodsky et al., 1999; Nishikawa et al., 2001; Taxis et al., 2003). Notably, a second class of cytosolic Hsp70s, Ssb1p and Ssb2p, are dispensable for VHL degradation (data not shown), suggesting a specific requirement for the Hsp70 Ssa1p in both VHL folding (Melville et al., 2003) and degradation (Figure 2C).

The Hsp70 Cochaperone STI1 Is Required for Degradation of Misfolded VHL

To better define the role of Hsp70 in misfolded VHL degradation, we next examined the requirement for several cofactors known to modulate the activity of Ssa1p (Frydman, 2001). The Hsp40 homolog Ydj1p, previously reported to contribute to the degradation of misfolded canavanyl polypeptides in yeast (Lee et al., 1996), is not required for VHL degradation (Figure 3A, top panel). Additionally, no defect in VHL degradation was observed in a strain with a temperature-sensitive allele of the essential Hsp40 homolog *SIS1* (data not shown), suggesting that VHL degradation may instead depend on another of the ~13 cytosolic Hsp40 homologs (Walsh et al., 2004).

One notable difference between the *SSA* and *SSB* Hsp70 classes is that *SSA*s contain a C-terminal motif, EEVD, which mediates interaction with tetratricopeptide (TPR) domain-containing proteins (Scheufler et al., 2000). Strikingly, the degradation of both misfolded VHL variants was markedly inhibited in cells lacking the TPR-containing Ssap cofactor *STI1* (Figure 3A, bottom panel; Figure S6Aiv).

Successful partitioning to the ubiquitination and degradation machinery requires that the polypeptide remain soluble. We therefore examined whether deletion of *STI1* blocks degradation by failing to prevent aggregation of misfolded VHL. In wt cells, most of the VHL protein was soluble and remained in the supernatant (Sn) of a high-speed centrifugation, with only a small fraction found in the insoluble pellet (Pel; Figure 3B). Importantly, no change in the solubility of VHL was observed in cells lacking Sti1p (Figure 3B). Because VHL remains soluble in the absence of Sti1p yet fails to be degraded, we conclude that the function of Sti1p in

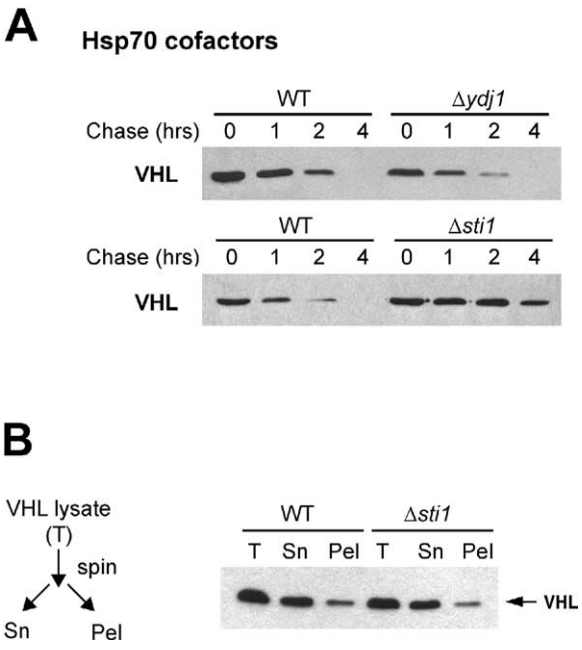

Figure 3. The Ssa1p Cochaperone Sti1p Is Required for VHL Degradation

(A) VHL/L158P degradation was examined in cells lacking the Hsp70 cofactors *YDJ1* and *STI1*.

(B) VHL solubility was assessed in wt and *Δsti1* yeast by comparing equivalent amounts of total (T), supernatant (Sn), and pellet (Pel) fractions (see Experimental Procedures) by SDS-PAGE and anti-VHL immunoblot.

degradation is not simply to maintain VHL solubility. This result suggests an active role for this cochaperone in VHL quality control.

Hsp90 Is Specifically Required for VHL Degradation but Is Dispensable for Folding

STI1, also called HOP (for Hsp70-Hsp90-organizing protein), has the unique ability to bridge a complex between Hsp70 and Hsp90 via specific interactions with its TPR domains (Scheufler et al., 2000). Thus, the requirement for Sti1p in VHL degradation may reflect a role not only for Hsp70 in this process but also for Hsp90. We therefore examined the contribution of Hsp90 to VHL degradation. Both L158P and wt VHL exhibited impaired degradation in cells carrying a temperature-sensitive allele of *HSP82* (*hsp82*^{ts} G170D; Nathan and Lindquist, 1995) at the nonpermissive temperature both under steady-state conditions (Figure 4A; see also Figures S5Aiii and S5Bii) and in pulse-chase analyses (Figure S2). Inefficient VHL degradation was also observed at 30°C in strains harboring constitutively defective point mutants of Hsp90, such as T101I and G313S (Nathan and Lindquist, 1995; Figure S3). Furthermore, Hsp90 inhibition with the ansamycin macbecin II also results in VHL stabilization, confirming a requirement for Hsp90 in VHL degradation (data not shown; see also Figure 4D below). Importantly, the ubiquitin-proteasome pathway was fully functional under our experimental conditions, as *hsp82* G170D cells supported the proteasome-dependent degradation of several substrates of this pathway at the nonpermissive temperature (Figure S4; for similar results, see also Taxis et al., 2003). For instance, the proteasome substrate firefly luciferase was efficiently degraded in *hsp82* G170D cells at 37°C (Figure S4). Furthermore, degradation of two misfolded TRiC/CCT interacting proteins, the folding-defective actin mutant E364K (Drummond et al., 1991) and nonnative CDC55, was unaffected when Hsp90 function was compromised (Figure S4). We conclude that the stabilization of VHL in *hsp82* G170D cells is not due to a general defect in proteasome-mediated degradation. Intriguingly, degradation of nonnative Tub3p, also a chaperonin substrate, was partially compromised in *hsp82* G170D cells at 37°C, suggesting that Hsp90 may play a role in quality control of other nonnative proteins (see also Discussion and Figure S7).

The requirement for Hsp90 in VHL degradation may in principle reflect a previously undescribed role for Hsp90 in VHL folding, which is coupled to assembly of the VBC complex (Feldman et al., 2003; Feldman et al., 1999; Melville et al., 2003). To test this possibility, we expressed VHL at the nonpermissive temperature in *hsp82* G170D cells already containing elongin BC and assessed its folding by several established assays (Figure 4B; Feldman et al., 2003; Feldman et al., 1999; Melville et al., 2003). Correctly folded VHL in VBC is resistant to mild protease digestion. Accordingly, VHL expressed in the absence of elongin BC is sensitive to protease treatment (Figure 4Ba, top panel). In contrast, VHL expressed in the presence of elongin BC was resistant to protease digestion in both wild-type and Hsp90-deficient cells (Figure 4Ba, center and bottom panels). The formation of correctly folded VBC was also monitored by coimmunoprecipitation of VHL with elongin BC (Figure 4Bb) and nondenaturing gel electrophoresis (Figure S5; Melville et al., 2003). Consistent with our protease-sensitivity data, both assays indicated that VBC assembly was not affected in *hsp82* G170D cells expressing VHL at either the permissive or nonpermissive temperature (Figure 4Bb, compare last two lanes). Importantly, as expected from these results, loss of Hsp90 function did not affect the solubility of VHL or its association with both Hsp70 and TRiC (data not shown).

Our findings that Hsp90 is dispensable for VHL folding, but required for VHL degradation, were confirmed by analysis of these processes in mammalian cells (Figures 4C and 4D). VBC assembly was analyzed in HEK cells as previously described (Feldman et al., 2003; Feldman et al., 1999; Melville et al., 2003), by coexpressing Flag-tagged wild-type VHL and elongin BC followed by VHL immunoprecipitation under stringent conditions (Figure 4C). The formation of tightly folded VBC was unaffected by incubation with the Hsp90-inhibiting drug geldanamycin (Figure 4C; Young et al., 2001). In contrast, geldanamycin treatment strongly inhibited the degradation of L158P VHL in a concentration-dependent manner (Figure 4D). Taken together, these experiments demonstrate that while VHL folding is independent of Hsp90, VHL degradation requires Hsp90 function. We conclude that Hsp90 plays a specific role in misfolded VHL degradation, raising the pos-

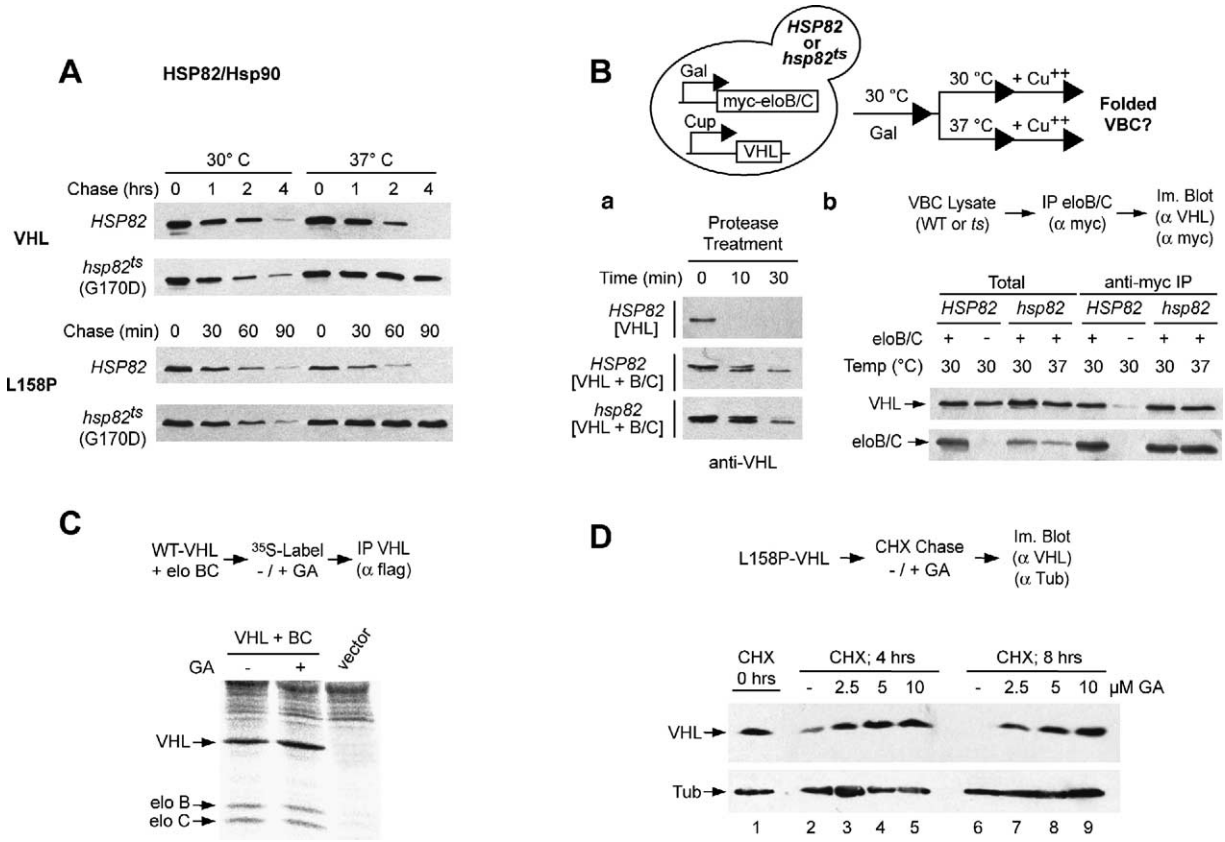

Figure 4. Hsp90 Is Specifically Required for VHL Degradation, but Not for VHL Folding, in Yeast and Mammalian Cells

(A) VHL/L158P degradation was examined in wt *HSP82* and temperature-sensitive *hsp82* (G170D) cells at both 30°C and 37°C.

(B) Scheme: experimental outline for assessing VHL folding in wt *HSP82* and temperature-sensitive *hsp82* cells. VHL expression was induced at either 30°C or 37°C in *hsp82* (G170D) cells already containing myc-tagged elongin BC. Formation of a correctly folded VBC complex was assessed using (Ba) a protease-protection assay (shown only for 37°C) and (Bb) an elongin BC affinity pull-down assay followed by the detection of VHL by immunoblot. VHL expressed in the absence of elongin BC is misfolded and serves as a negative control in both assays.

(C) The Hsp90 inhibitor geldanamycin (GA) does not affect VBC assembly in mammalian cells. HEK293 cells coexpressing Flag-tagged wild-type VHL and elongin BC or containing the backbone vector plasmid (vector) were incubated with or without 5 μM GA prior to [^{35}S]methionine labeling and immunoprecipitation with anti-Flag antibodies.

(D) The Hsp90 inhibitor GA inhibits degradation of misfolded VHL in mammalian cells. HEK293 cells expressing L158P VHL were chased with cycloheximide in the absence (lanes 2 and 6) or presence of increasing concentrations of GA (lanes 3–5 and 7–9). At the indicated chase times, L158P VHL and tubulin (which serves as a loading control) were detected by immunoblot analysis.

sibility that an Hsp70-Sti1p-Hsp90 complex functions in cytosolic quality control.

A Specific Hsp90-Containing Complex Mediates Degradation of Misfolded VHL

We next determined whether misfolded VHL interacts with Hsp70 and Hsp90 in vivo. Immunoblot analysis of VHL immunoprecipitations confirmed its association with the Hsp70 Ssa1p (Figure 5A; Melville et al., 2003) and established its association with Hsp90 (Figure 5A). Analysis of VHL bound proteins by mass spectrometry confirmed these results (data not shown) and also revealed an association with the Hsp110 chaperone Sse1p, which is genetically and biochemically linked to Hsp90 and *STI1* (Goeckeler et al., 2002; Liu et al., 1999). Immunoblot analysis confirmed this interaction (Figure 5A), indicating that additional Hsp90 cofactors might have a role in the degradation of misfolded VHL. Of note, no VHL interaction was observed for Ydj1p, which is not involved in VHL folding or degradation, nor with actin, which served as a specificity control (Figure 5A).

Hsp90 is the central component of a dynamic chaperone machine whose best-characterized role is in the maturation of a subset of substrates, also called "client" proteins, including kinases and steroid hormone receptors (Picard, 2002; Young et al., 2001). It is proposed that these folding clients proceed from "early" Hsp90 complexes containing Hsp70, Sti1p/HOP, Sse1p, and Hsp90 to "late" or "mature" complexes containing Hsp90 and a different complement of cochaperones, among them peptidyl-prolyl isomerases and Sba1p/p23 (Figure 5B; Picard, 2002; Young et al., 2001). Importantly, formation of the late complex(es) is essential for folding of client substrate proteins. We next examined the role of these Hsp90 complexes in VHL degradation. As observed for *STI1*, deletion of *SSE1* resulted in strong stabilization of misfolded VHL (Figure 5B, Early Complex), consistent with our finding that Sse1p asso-

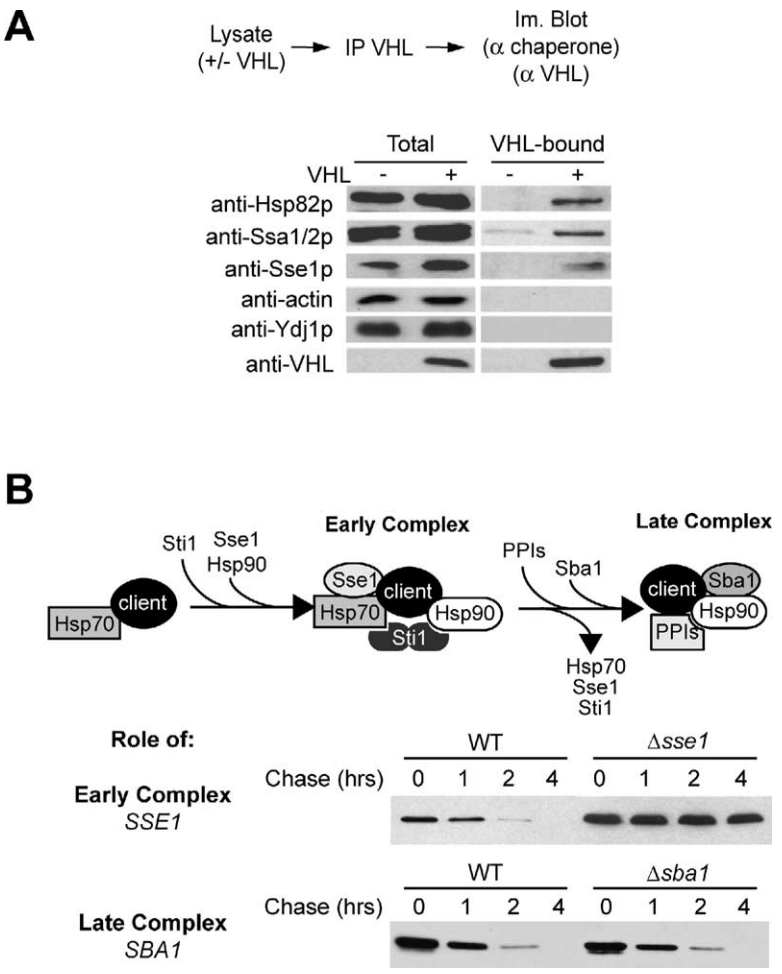

Figure 5. An "Early" Hsp90 Complex Participates in VHL Degradation

(A) Immunoprecipitation of VHL was followed by detection of associated Ssa1/2p, Hsp90, Sse1p, and Ydj1p with the appropriate antisera. Actin serves as a specificity control for the immunoprecipitation.

(B) Scheme: sequential formation of Hsp90 complexes in client-protein folding. The contribution of Hsp90 subcomplexes to VHL degradation was examined in yeast lacking *SSE1* (an early complex component) or *SBA1* (a late complex component) and in the isogenic wt strains.

ciates with VHL. In contrast, *SBA1* deletion had no effect on VHL degradation (Figure 5B, Late Complex). Thus, while components of the early Hsp90 complex, including *STI1*, *SSE1*, and Hsp70, are required for VHL degradation, components of the late complex, such as Sba1p/p23 and Aha1p (data not shown), are dispensable, suggesting that quality control of misfolded VHL is specifically dependent on an early Hsp90 complex (Figure 6).

Discussion

A major unexplored question in cell biology is how the cell selects misfolded cytosolic proteins and targets them for elimination. Triage decisions must balance the need to protect nonnative newly translated proteins that have not yet reached their fully folded state with the requirement to prevent the accumulation of aggregation-prone toxic species. Here we investigated how the cellular chaperone machinery participates in quality control of a physiologically relevant misfolded cytosolic protein. The availability of folding-defective variants of a cellular protein with a well-characterized chaperone-dependent folding pathway, the VHL tumor suppressor, allowed us to distinguish between possible models for chaperone function in quality control. Previous studies using artificial or damaged substrates that were either grossly misfolded and aggregation prone or poorly characterized with regard to their chaperone interactions led to a model whereby chaperones play a passive, nonspecific holding function in degradation, merely maintaining nonnative polypeptides in a soluble state. A more complex role for chaperones in cytosolic quality control emerges from our in vivo analysis of misfolded VHL degradation, which indicates that specific, rather than stochastic, chaperone pathways govern its folding and degradation (Figure 6). Of the two chaperones required for VHL folding, TRiC/CCT and Hsp70 (Ssa1p), only Ssa1p is required for degradation, while TRiC/CCT function is dispensable (Figure 2). Strikingly, VHL degradation specifically requires another chaperone, Hsp90, which does not participate in VHL folding (Figure 4). Importantly, VHL stabilization in Hsp90- or Sti1-deficient cells is not caused by aggregation (Figure 3B and data not shown) but rather results from the loss of specific chaperone interactions with Hsp90 and a subset of Hsp90 cofactors. Since VHL is still soluble and associated with chaperones, including Hsp70 and TRiC, in *Δsti1* or Hsp90-deficient cells where degradation is prevented, we conclude that binding to TRiC or Hsp70 does not suffice to target misfolded VHL for degradation. Furthermore, because loss of Hsp90 func-

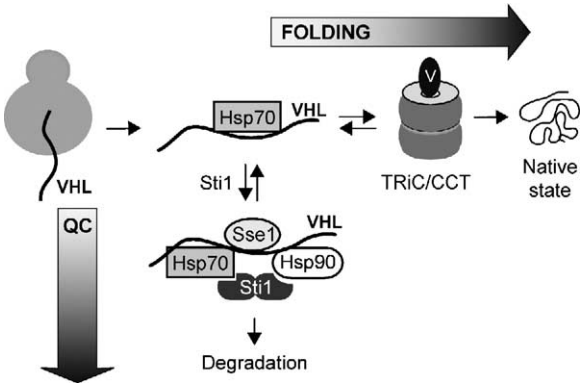

Figure 6. Two Distinct Chaperone Pathways Mediate VHL Folding and VHL Quality Control

Newly synthesized VHL is folded with the assistance of Hsp70 and TRiC. Folding-defective VHL is transferred to an early Hsp90 complex with the assistance of Sti1p. An Hsp90 subcomplex containing Hsp70 (Ssa1p), Sti1p, Sse1p, and Hsp90 facilitates the recognition and degradation of VHL by the ubiquitin-proteasome pathway.

tion blocks VHL degradation, our data indicate that Hsp90 is not merely engaging VHL in a futile folding cycle but is actually actively required for degradation. These findings are inconsistent with proposed models for cytosolic quality control whereby chaperones function only to maintain polypeptide solubility and passively facilitate partitioning to the degradation machinery. Instead, it appears that certain chaperones play an active and specific role in the triage process.

The specific requirement for Hsp90 in the degradation of a protein that is not one of its folding clients is surprising. Our data suggest that early Hsp90 complexes not only are intermediates leading to formation of late Hsp90 complexes during client-protein folding but may also have an independent function in quality control. Accordingly, we propose that Hsp90 function is not limited to folding a restricted subset of client proteins (Young et al., 2001) but may also function in quality control of nonclient, cytosolic proteins. How may Hsp90 mediate protein quality control? A number of mechanisms are consistent with our findings. For instance, Hsp90 may directly recruit specific ubiquitination components, as observed in mammalian cells for the ubiquitin ligase CHIP, which interacts with both Hsp90 and Hsp70 (Ballinger et al., 1999; Connell et al., 2001). Although no CHIP homologs exist in yeast and the role of CHIP in misfolded-protein degradation is not yet well established, it is possible that additional ubiquitin ligases interact with an early Hsp90 complex (see for instance Bansal et al., 2004). Alternatively, a Hsp90 complex may facilitate direct delivery of the substrate to the proteasome since Hsp90 has been shown to interact with proteasome complexes (Verma et al., 2000). Finally, it is also possible that Hsp90 action is uniquely required to generate a specific conformation of the misfolded substrate that can be subsequently recognized by a ubiquitin ligase involved in quality control. Future studies should clarify the precise mechanism by which Hsp90 promotes VHL quality control.

The idea that Hsp90 and some of its cofactors have an additional function in quality control of misfolded proteins is consistent with the strong upregulation of the Hsp90 system by amino acid analogs that generate misfolded proteins or during environmental stress (Nicolet and Craig, 1989), with the colocalization of Hsp90 with misfolded protein aggregates (Dou et al., 2003; Mitsui et al., 2002) and with the observation that Hsp90 enhances the proteasome-mediated antigen processing of ovalbumin (Yamano et al., 2002). Interestingly, while inhibition of Hsp90 by ansamycins, which abolishes folding of client proteins, leads to degradation of the Hsp90 bound client (Young et al., 2001), we find that inhibition of Hsp90 with ansamycins blocks the degradation of VHL (Figure 4D). Although the substrate spectrum of most cellular chaperones, including Hsp90, remains ill defined even for folding substrates, it is likely that our findings for VHL also apply to the quality control of other cellular proteins. For instance, the proteasome-mediated degradation of both CYP2E1 (cytochrome P450 2E1) and apolipoprotein B is also blocked upon treatment with Hsp90-inhibiting ansamycin drugs (Goasduff and Cederbaum, 2000; Gusarova et al., 2001). Impairing Hsp90 function enhances ApoB maturation (Gusarova et al., 2001), suggesting that, similar to VHL, Hsp90 is also required for ApoB degradation but dispensable for folding. On the other hand, the degradation of nonnative variants of actin and CDC55 is not compromised in the absence of Hsp90 function (Figure S4), indicating the existence of alternative pathways of cytosolic quality control. Preliminary experiments comparing wild-type and *hsp82* cells in pulse-chase analyses to determine what percentage of rapidly turned-over amino acid analog-containing proteins are stabilized by Hsp90 deficiency demonstrated an ~20% reduction of total degradation when Hsp90 function was compromised (Figure S7). Since the pulse-chase experiment looks at the degradation of all rapidly turned-over polypeptides, whether or not they are found in the ER, mitochondria, or the cytoplasm, the fraction of cytosolic proteins stabilized in the Hsp90 mutants may be higher than this rough analysis indicates. Thus, Hsp90 is important for the degradation of a significant fraction of, but not all, misfolded polypeptides. It is likely that cells contain several chaperone pathways leading to the degradation of distinct classes of misfolded proteins, similar to what is observed for chaperone-mediated protein folding, where different chaperone pathways mediate the folding of different classes of proteins. This might be expected, given the critical importance of ensuring efficient protein quality control for cell viability and the fact that, upon misfolding, different structural classes of proteins may require distinct cellular components for efficient recognition and elimination. An important question for future studies will be to define the spectrum of proteins that require Hsp90 for quality control.

Our analysis of the chaperone pathways required for folding and quality control of the VHL tumor suppressor provides novel insights into the logic underlying triage decisions in the eukaryotic cytosol. The identification of two distinct pathways of chaperone interactions for VHL (Figure 6), one leading to folding and one to degradation, suggests that the fate of a given cytosolic protein may be controlled by a hierarchy of chaperone in-

teractions. In the case of VHL, cotranslational coupling mechanisms may establish an initial association with TRiC (Thulasiraman et al., 1999), resulting in a chaperone hierarchy that favors folding over degradation. However, failure to fold may allow subsequent association with the Hsp90 complex, leading to degradation. Consistent with this model, we find that the *tcp1-2* mutation, which destabilizes the TRiC complex, resulting in substrate binding defects (Camasses et al., 2003), slightly enhances the degradation rate of VHL (Figure 2B), suggesting that the folding and degradation pathways may be in a dynamic equilibrium. In principle, the fate of newly translated VHL may be governed by the affinity and binding kinetics of its folding intermediates to the chaperone systems in each pathway. Of note, Hsp90 has been proposed to recognize structured folding intermediates (Picard, 2002; Young et al., 2001), such as those that might result from failed folding attempts. Accordingly, Hsp90 may recognize a conformation of VHL that is not reached under productive folding conditions; that might be why, in the presence of elongin BC, TRiC-mediated folding has precedence over Hsp90 binding and degradation. Hsp70, uniquely required for both folding and degradation, may play a key role in the decision-making process, possibly in cooperation with the Hsp70-Hsp90-bridging cochaperone Sti1p/HOP. Strikingly, similar principles may be at work during quality control of glycosylated proteins in the ER (Ellgaard and Helenius, 2003), where association with the chaperone calnexin is conducive to folding, while transfer to EDEM signals the commitment to degradation. Our findings for VHL may reflect a more general principle of protein triage and provide a conceptual framework to understand how the cell determines that a nonnative polypeptide is a "lost cause."

Experimental Procedures

Yeast Media, Plasmids, and Strains

Yeast media preparation, growth, transformations, and manipulations were performed according to standard protocols (Adams et al., 1997).

His_6-VHL and His_6-L158P were cloned into pESC (GAL1 *URA3*; Stratagene, La Jolla, California). The pESC plasmid expressing elongin B and elongin C is described elsewhere (Melville et al., 2003).

The yeast strains used in this study are as follows: wt *CIM3* (YPG499; MATa *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101*) and *cim3-1* (CMY762; *ura3-52 leu2-Δ1 his3-Δ200 cim3-1*) (Ghislain et al., 1993); MHY501 (MATα *his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*) and the isogenic mutant strains MHY508 *(ubc4::HIS3 ubc5::LEU2*) and MHY570 (*ubc4::TRP1 ubc5::LEU2 ubc6::HIS3 ubc7::LEU2*) (Chen et al., 1993); wt *TCP1* (DUY558; MATα *leu2-3, 112 ura3-52 trp1-7 tcp1::LEU2 p-TCP1* [*TRP1*]) and *tcp1-2* (DUY326; MATα *leu2-3, 112 ura3-52 trp1-7 tcp1::LEU2 p-tcp1-2* [*TRP1*]) (Ursic et al., 1994); *SSA1* (JN516; MATα *ura3-52 leu2-3 his3-11, 15 trp1-Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*) and *ssa1-45* (JB67; MATα *ura3-52 leu2-3 his3-11, 15 trp1-Δ1 lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*) (Becker et al., 1996); wt *Δsti1*, *Δydj1*, and *Δsse1* (MATa/MATα *orfΔ::kanMX4/orfΔ::kanMX4 ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ1/his3Δ1 met15Δ0/MET15 lys2Δ0/LYS2* (*Saccharomyces* Genome Project; Winzeler et al., 1999); wt *HSP82* (iP82a; MATa *ura3-1 leu2-3,12 his3-11,15 trp1-1 can1-100 ade2-1 hsc82::LEU2 hsp82::LEU2 his3-11, 15::GPD-HSP82-HIS3*) and *hsp82^{G170D}* (iG170Da; MATa *ura3-1 leu2-3,12 his3-11,15 trp1-1 can1-100 ade2-1 hsc82::LEU2 hsp82::LEU2 his3-11, 15::GPD-hsp82^{G170D}-HIS3*) (also iG313Sa, same genotype; Nathan and Lindquist, 1995); wt *SBA1* (YNK100; MATα *pdr5-101 ura3*) and *Δsba1* (YNK233; MATa *pdr5-101 ura3 sba1::HIS3*) (Bohen, 1998); W303 (MATa *ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1*) and W303 *Δelc1* (MATa *ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 elc1::KAN^r*) (Jackson et al., 2000).

Cell Culture and Transfection

HEK293 cells cultured in DMEM/5% FCS (Gibco) were transfected with 8–10 μg of plasmid DNA using calcium phosphate as described (Feldman et al., 2003).

Mammalian expression plasmids for wt VHL, L158P VHL, elongin B, and elongin C were described previously (Feldman et al., 2003).

Additional Reagents

The Hsp90-inhibiting ansamycins macbecin II and geldanamycin (GA) were obtained from the NCI Drug Synthesis and Chemistry Branch and were solubilized in DMSO (Sigma) prior to use.

VHL Degradation

Steady-State Degradation Assay in Yeast

VHL expression was induced by galactose addition to cells at a very low density. Next the cells were grown at 30°C for ~16 hr to an OD_{600} of 0.5–0.8, diluted to an OD_{600} of 0.2 in 50 ml of YPD to repress VHL expression (time 0), and further incubated at 30°C or 37°C. Samples were removed at indicated time points, harvested, washed once with sterile double-distilled H_2O, and frozen in liquid nitrogen. Cell pellets were stored at –20°C until extract preparation. Extracts were prepared by 10 min agitation with glass beads on a turbo-mix vortex (Fisher Scientific, Pittsburgh) in lysis buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 0.5% SDS, 1 mM dithiothreitol [DTT], 1 mM phenylmethyl-sulfonyl fluoride [PMSF], and 1 μg/ml pepstatin-A), heated at 65°C for 10 min, and spun at 16,000 × g for 10 min at 4°C. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, Illinois). Samples from each time point containing identical amounts of protein (15 μg for wt VHL and 45 μg for L158P VHL) were separated on 12% SDS-PAGE gels and transferred to Protran nitrocellulose (0.45 μM pore size; Schleicher & Schuell Inc., Keene, New Hampshire). VHL was detected using anti-VHL primary antibody as described (Melville et al., 2003). As a loading control, actin was detected using anti-actin primary antibody (for example, see Figure S3; Melville et al., 2003). Actin, luciferase, GST-CDC55, and TUB3-myc steady-state degradation assays were conducted exactly as described for the VHL steady-state degradation assay, with the exception that the appropriate time courses and antisera for detection were used.

Steady-State Degradation Assay in Mammalian Cells

The effect of GA on the degradation of L158P VHL was determined using a cycloheximide chase. Cells were incubated with 1 mM cycloheximide together with increasing concentrations of GA or an equivalent volume of DMSO. At the indicated time points, cells were lysed in buffer M (25 mM Tris-HCl [pH 7.4], 5 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 1% Triton X-100, 1 mM NaN_3, 2 mM deoxyglucose) and clarified by centrifugation at 20,000 × g for 20 min. Fifty micrograms of each resulting cell extract was analyzed by SDS-PAGE and immunoblot. VHL was detected using anti-VHL primary antibody; as a loading control, tubulin was visualized using a monoclonal anti-β-tubulin antibody (Amersham Pharmacia Biotech, Piscataway, New Jersey).

Metabolic Labeling and Pulse-Chase Experiments

hsp82 (G170D) yeast expressing VHL were grown in SC Gal media to midlogarithmic phase. Cells were harvested, washed twice with –Met Gal media, then resuspended to 5 ODs/ml in –Met Gal. The cells were then incubated at 30°C or 37°C with gentle shaking (~200 rpm) for 30 min. 0.3 mCi of [^{35}S]methionine/cysteine was added, and cells were labeled for 5 min. Cells were collected, washed, and resuspended in YPD supplemented with 0.5 mg/ml cycloheximide and 0.5 mg/ml methionine. Four hundred microliters of cells was removed to 400 μl ice-cold 0.1 M NaN_3 at the indicated times. Yeast cell pellets were lysed as described above, and 75 μg of each time point was immunoprecipitated with anti-VHL antisera (or control mouse IgG) and protein G-Sepharose. Following SDS-

PAGE, the gels were dried and exposed to a phosphorimager cassette for 3 days.

For experiments in Figure 4C and Figure S1, cells were labeled for 12 hr in MEM containing 0.2 mCi [^{35}S]methionine/cysteine mix (NEN) and 1% FCS. Where indicated, cells were chased into media containing DMEM/5% FCS and 0.5 mg/ml cycloheximide then lysed as above. Cell extracts (50 μg) were analyzed using a stringent immunoprecipitation protocol as described (Feldman et al., 2003). Equivalent amounts of nonimmune antibodies were used in control immunoprecipitations.

VHL Solubility Assay

Cells expressing VHL were grown at 30°C or 37°C (for temperature-sensitive strains; other strains were grown only at 30°C), harvested, washed once with sterile double-distilled H_2O, and resuspended in 500 μl of native yeast lysis buffer (20 mM HEPES [pH 7.4], 200 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin-A). Lysates were prepared by bead beating (10 min at 4°C) and clarified by centrifugation at 16,000 × g for 10 min at 4°C. One hundred and fifty microliters of this supernatant was set aside as total protein. One hundred and fifty microliters was spun in a Beckman T120.1 rotor at 350,000 × g for 15 min at 4°C. This supernatant was removed and designated the soluble fraction. The pellet was resolubilized by heating in 200 μl 1× SDS sample buffer. Fifty microliters of 4× SDS sample buffer was added to the total-protein and soluble-fraction samples. Equal amounts of each fraction were resolved by SDS-PAGE followed by immunoblot analysis with anti-VHL antisera.

VHL Folding Assays

Yeast containing a single wild-type allele of Hsp90 (wt 90) or a temperature-sensitive point mutant allele (G170D) were transformed with pCu426-His$_6$-VHL (*URA3*) and pESC-myc-elongin B/myc-elongin C (*TRP1*). To induce expression of elongin BC, cells were grown overnight in SC Gal Raf (lacking uracil and tryptophan). Cultures were split, placed at 30°C and 37°C, and allowed to equilibrate for 1 hr. Then, VHL was induced for 2 hr by addition of 300 μM $CuSO_4$. Cells were harvested, washed once with sterile double-distilled H_2O, resuspended in VBC lysis buffer (25 mM Tris [pH 8.0], 75 mM ammonium sulfate, 2.5 mM EDTA, 2.5% glycerol, 1 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin-A) and lysed as above. Lysates were clarified by centrifugation at 16,000 × g for 30 min at 4°C, and protein concentration was determined by the BCA method.

VHL folding was examined as described (Melville et al., 2003; Feldman et al., 2003). To examine VBC assembly, 250 μg of each lysate was immunoprecipitated with 2 μl of anti-myc antibody (Covance Inc., Princeton, New Jersey) and 40 μl of protein G slurry (Amersham Pharmacia Biotech). The protein G beads were washed three times with wash buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 10% glycerol, 0.5% Triton X-100). Immunoprecipitated proteins were analyzed by SDS-PAGE (15%), anti-VHL immunoblot to detect VHL, and anti-myc immunoblot to detect elongin BC. To examine the thermolysin sensitivity of VHL, lysates were buffer exchanged into thermolysin buffer (50 mM Tris [pH 8.0], 10 mM $CaCl_2$, 50 mM KCl, and 5% glycerol) using NAP 5 columns (Amersham Pharmacia Biotech). Reactions containing 120 μg lysate and 0.3 mg/ml thermolysin in a total volume of 180 μl were incubated on ice for the indicated times and analyzed by SDS-PAGE followed by immunoblotting with anti-VHL antisera.

Supplemental Data

Supplemental Data include seven figures and are available with this article online at http://www.cell.com/cgi/content/full/121/5/739/DC1/.

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