Involvement of Valosin-containing Protein, an ATPase Co-purified with IκBα and 26 S Proteasome, in Ubiquitin-Proteasome-mediated Degradation of IκBα*

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The inactivation of the prototype NF-κB inhibitor, IκBα, occurs through a series of ordered processes including phosphorylation, ubiquitin conjugation, and proteasome-mediated degradation. We identify valosin-containing protein (VCP), an AAA (ATPases associated with a variety of cellular activities) family member, that co-precipitates with IκBα immune complexes. The ubiquitinated IκBα conjugates readily associate with VCP both in vivo and in vitro, and this complex appears dissociated from NF-κB. In ultracentrifugation analysis, physically associated VCP and ubiquitinated IκBα complexes sediment in the 19 S fractions, while the unmodified IκBα sediments in the 4.5 S fraction in VCP. Phosphorylation and ubiquitination of IκBα are critical for VCP binding, which in turn is necessary but not sufficient for IκBα degradation; while the N-terminal domain of IκBα is required in all three reactions, both N- and C-terminal domains are required in degradation. Further, VCP co-purifies with the 26 S proteasome on two-dimensional gels and co-immunoprecipitates with subunits of the 26 S proteasome. Our results suggest that VCP may provide a physical and functional link between IκBα and the 26 S proteasome and play an important role in the proteasome-mediated degradation of IκBα.

Transcription factor NF-κB is involved in a large variety of processes, such as cell growth, transcriptional regulation, immune, inflammatory, and stress responses (reviewed in Refs. 1–4). NF-κB is a homo- or heterodimer consisting of various combinations of the family members, including NFκB1 (p50 and precursor p105), c-Rel, RelA, NFκB2 (p52 and precursor p100), RelB, and Drosophila proteins Dorsal and Dif. Unlike many other transcription factors that are localized in the nucleus, the NF-κB dimeric factor is sequestered in the cytoplasm of most cells through binding to a family of inhibitor proteins, IκB. In response to extracellular stimuli, the inhibitors are partially or entirely degraded, thus liberating the DNA-binding dimer for translocation to the nucleus. The 16B family contains IκBα, IκBβ, IκBγ, Bcl-3, the precursor proteins p105 and p100, and the Drosophila protein Cactus. All members of the IκB family contain 5–8 ankyrin motifs, thought to be involved in protein-protein interactions. It has been shown that when the precursor protein p105 is involved as the inhibitor, the processing from p105 to the active p50 occurs through the ubiquitin-dependent proteasome (Ub-Pr)1 pathway, which degrades the C-terminal ankyrin-containing domain of p105 (5, 6).2 For the prototype complex that contains p50, p65, and IκBα, upon stimulation the entire NF-κB complex becomes hyperphosphorylated. The induced phosphorylation of IκBα does not lead to its immediate dissociation from the complex; rather, it signals for rapid IκBα degradation, thus liberating the active p50/p65 dimer for translocation to the nucleus (7–15). We and others showed that the degradation of IκBα is sensitive to proteasome inhibitors and is ubiquitin-dependent. Recently, it was further shown that signal-induced phosphorylation precedes IκBα degradation and targets IκBα to the Ub-Pr pathway (7–15). It was proposed that the inactivation of IκBα occurs through a series of ordered processes including phosphorylation, ATP-dependent multiple ubiquitin conjugation, and proteasome-mediated proteolysis. However, the link between ubiquitination and proteasome-mediated degradation remains unclear.

The extralysosomal, energy-dependent Ub-Pr pathway is a major mechanism used to regulate many critical cellular proteins that must be rapidly destroyed for normal growth and metabolism (reviewed in Refs. 16–21). The rapidly growing list of the substrates for the Ub-Pr pathway includes mitotic cyclins, G1 cyclins, cyclin-dependent kinase inhibitors p27, Sic1 protein, proto-oncogene products p53, c-Myc, c-Jun, and c-Mos, NFκB inhibitors, yeast MATα2 transcription factor, major histocompatibility complex molecules, and others. The Ub-Pr proteolytic pathway is ATP-dependent and present in both cytoplasm and nucleus. The pathway consists of two distinct, sequential steps. The target protein is first conjugated with multiple ubiquitin (Ub) molecules that mark the substrate for destruction. The Ub-tagged target is then translocated to (probably with the help of molecular chaperones) and degraded by a large protease complex with an apparent sedimentation coefficient of 26 S. The 26 S proteasome is a multisubunit complex, consisting of a central cylinder-shaped 20 S multicatalytic proteasine core and a 19 S cap-like regulatory complex attached to each end of the cylinder. The terminal cap structure consists of at least 18 distinct subunits with molecular masses of 35–110 kDa and has ATPase and ubiquitin conjugate binding activities. It is presumed that ATP hydrolysis by the 19 S complex provides the energy needed for the chaperoning and unfolding

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1 The abbreviations used are: Ub-Pr, ubiquitin-dependent proteasome; Ub, ubiquitin; Ub-IκBα, ubiquitinated IκBα; IP, immunoprecipitation; RIPA, radioimmuno precipitation; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; ATP-5′, adenosine 5′-O-(thiotriphosphate); VCP, valosin-containing protein.

2 C.-C. H. Li, unpublished observations.
of substrates degraded within the proteasome cylinder. VCP (22–24), the mammalian homolog of the cell division cycle protein Cdc48p in yeast and p97 in Xenopus, is a member of a recently identified AAA family (reviewed in Ref. 25). The family members are characterized by having ATPase domain(s) with striking sequence similarities and having ring structures consisting of homooligomers. Despite the high sequence and structural homology, these proteins unexpectedly are implicated in a large variety of seemingly unrelated biological functions. These functions reviewed in Ref. 25 include control of cell division cycle, T cell activation (23, 24), membrane fusion (26–29), and vesicle-mediated transport, peroxisome assembly and biogenesis, gene expressions, and the Ub-Pr degradation pathways (30). Furthermore, at least six family members have been identified as subunits of the 26 S proteasome (subunits 4, 6, 7, 8, 10, and TBP-1) (31–36), and it is likely that other family members will fill this function as well (31, 37). The apparent paradox between the striking sequence homology and the large diversity of functions in this family suggests that these proteins have a basic and critical role in cellular function, and the involvement in many other functions may be indirect.

During the course of studying the molecular mechanisms involved in IxBo degradation, we detected VCP physically associated with IxBo complexes both in vitro and in vivo. In this report, we demonstrate that VCP is involved in the proteasome-mediated degradation of IxBo, and VCP is co-purified with the 26 S proteasome. Consistent with this hypothesis, VCP indeed has in vitro ATPase activity and an apparent sedimentation coefficient of 19 S (Ref. 24 and this study), the same as that of the regulatory complex of the 26 S proteasome. Consistent with this hypothesis, the 26 S proteasome-depleted (Pr−2) proteasomes were isolated from human red blood cells (38), which were essentially the same as the degradation assay except that the proteasome-depleted (Pr−2) proteasomes were generated by slightly modified Ub-conjugation assays (6, 8), which were essentially the same as the degradation assay except that the proteasome-depleted (Pr−2) proteasomes were isolated from human red blood cells (38).

**Materials and Methods**

Cell Lines and Culture Conditions—Two interchangeably used human B cell lines, DB (38) and CA46 (39), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 munits/ml streptomycin, and 50 μg/ml streptomycin.

Immunoprecipitation (IP) and Western (Immuno-) Blot Analyses—Both analyses were performed as described previously (9, 40, 41) with minor modifications. Cells were labeled with 0.1 μCi/ml (1000 Ci/mmol) [35S]methionine/cysteine (ICN) at a density of 5 × 10^6/ml for approximately 16 h, washed twice with phosphate-buffered saline, and lysed in RIPA buffer (20 mM Tris/HCl, pH 7.6, 2 mM EDTA, 150 mM NaCl, 1% deoxycholate X-100) containing protease inhibitors (1% aprotinin, 70 μg/ml phenylmethylsulfonyl fluoride, 40 μg/ml PMSF-Phe-ChlCI, 5 μg/ml Tos-Lys-ChlCl, 5 μg/ml Leupeptin). The lysates were clarified by centrifugation at 12,000 × g for 30 min and incubated with antisera. The immune complexes were collected with Protein A-Sepharose beads, washed with RIPA buffer, boiled, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), electrophoretically transferred onto polyvinylpyrrolidone membranes, and analyzed by autoradiography. IPs shown in Figs. 1A and 2B were conducted in Buffer I (1% Nonidet P-40, 1% bovine serum albumin in phosphate-buffered saline) containing protease inhibitors, 100 μM calpain inhibitor I, and 10 μM iodoacetamide, and the precipitates were washed with Buffer I without bovine serum albumin. For reimmunoprecipitation experiments, the immune complexes isolated from the first IP were boiled in RIPA containing 1% SDS, diluted, and subjected to the second IP. For immunoblot analysis, equal amounts of protein or immune complexes were resolved by SDS-PAGE and transferred onto membranes. The membrane was blocked, washed, and incubated with antisera (typically at 1:3,000), followed by reaction with peroxidase-conjugated anti-rabbit immunoglobulin serum (Boehringer Mannheim), and developed by the enhanced chemiluminescence detection system (ECL) (Amersham Corp.). When serial blotting analyses were performed, previous reactivity was stripped off from the membrane.

**Antisera—**All antisera used in this study is polyclonal rabbit sera. Anti-IxBo-1 and anti-IxBo-2 (40, 9) were independently generated against synthetic peptide corresponding to residues 300–317 of the human IxBo sequence (42). Anti-IxBo-3 (SC-847) and -5 (SC-371) were purchased from Santa Cruz Biotechnology, Inc., and anti-IxBo-4 (66–494) was purchased from Upstate Biotechnology, Inc. Anti-VCP-1 and anti-VCP-2, raised against peptides of residues 792–806 and 20–40 of murine VCP (23), respectively, were kindly provided by L. Samelson (NIH). Anti-VCP-3, -4, -5, -6, -7, and -8 were generated against glutathione-S-transferase (GST)-full-length and recombinant proteins 900–906, 721–734, 184–197, 240–253, and 167–180 of murine VCP (23), respectively. Anti-Ub polyclonal antisera were purchased from either Sigma or Biogenesis or obtained from M. Rechsteiner (University of Utah, Salt Lake City, Utah) (43). Anti-sera to subunits 4 and 5 and to the 26 S proteasome were kindly provided by M. Rechsteiner (43, 44).

**Protein Sequencing—**Well resolved protein bands were sliced from Coomassie Blue-stained gels and subjected to in-gel partial VCP digestion (45). The proteolytic fragments were resolved by 15% SDS-PAGE, transferred onto a polyvinylpyrrolidone membrane, and stained with Coomassie Blue. The well resolved bands were sliced and subjected to N-terminal peptide sequencing using an ABI 494A sequencer.

**In Vitro Association—**IxBo (7) or VCP (23) was synthesized in a coupled in vitro transcription and translation reaction from a rabbit reticulocyte lysate system (Promega) in the presence of [35S]cysteine (for IxBo) or [35S]methionine (for VCP). GST-IxBo expression plasmid was constructed by inserting the EcoRI fragment of IxBo (7) into pGEX-4T-2 vector (Pharmacia Biotech Inc.) (9). The GST-VCP (24) or GST-IxBo fusion proteins were prepared according to the manufacturer (Pharmacia). Glutathione-Sepharose beads containing GST or GST fusion proteins were incubated with 35S-labeled in vitro translated protein or unlabeled B cell lysates in a total volume of 225 μl of binding buffer (5 mm Tris, pH 7.6, 50 mm NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol including protease inhibitors). After incubation for 2 h at 4 °C and three washes with binding buffer, the bound products were analyzed by SDS-PAGE followed by Western transfer, autoradiography, and immunoblotting.

**Glycerol Gradient Sedimentation Centrifugation—**Velocity sedimentation centrifugation was carried out in 10–40% glycerol gradients in a total volume of 13 ml of 12 ml Tris-HCl, pH 7.5, 50 mm NaCl, 2 mM CaCl2, 1 mM MgCl2. Freshly prepared CA46 cell lysate in a volume of 200 μl was loaded on the gradient and centrifuged at 4 °C, in an SW41 rotor at 36,000 rpm for 16 h. Fractions of 0.5 ml were collected and analyzed by immunoblotting or IP. Protein markers were centrifuged in separate tubes and included thyroglobulin (19 k), immunoglobulin G (7 k), and bovine serum globulin (45.5 k).

**Proteasome Purification—**Cytosolic fractions were prepared by lysing cells in a hypotonic buffer (5 mM Hepes, pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol) on ice for 10 min. The lysates were clarified by low speed centrifugation for 10 min. The supernatant was further clarified at 100,000 g for 1–2 h, and the supernatant (S100), was stored in aliquots at −70 °C. Proteasome-enriched (Pr−) and proteasome-depleted (Pr+) fractions were extracted from CA46 cells as described by Palombella et al. (5). The highly purified (26 S-1) and the partially purified (26 S-2) proteasomes were isolated from human red blood cells as described (46).

**In Vitro Transfections—**In vitro transfections of IxBo encoding amino acids 37–317, and 1–242 were conducted as described by Brockman et al. (10). The deletion mutant encoding amino acids 23–317 was constructed by polymerase chain reaction using specific oligonucleotide primers (5′-GGGAAACCTCTCGTCGCGCCCATGCGGTACTGGACAGGCCG-3′ and 5′-GGTGTAGATGATACGTCGCCGCTCCTGG-3′) and the wild-type IxBo cDNA (7) as a template. Amplified product was purified and cloned into pcRII vector (TA cloning kit, Invitrogen). The specific mutants, S32A/S36A, and K21R/K22R, were kindly provided by D. Ballard (12).

**In Vitro Assays—**The wild-type and mutants of IxBo were synthesized in reticulocyte lysate-based in vitro transcription/translation system (Promega) in the presence of [35S]cysteine and used as the substrates in the Ub-Pr degradation assay (9). S100 was extracted from CA46 cells (8) and used as enzyme source. Master reaction mixture containing 5 μl of substrate, 8 μg of dialyzed ubiquitin (Sigma), 12 mM Tris-HCl, pH 7.5, 60 mM KCl, 3.5 mM MgCl2, 5 mM CaCl2, 1 mM dithiothreitol, and 1 mM ATP was prepared and aliquoted into four tubes on ice. At various time points, 50 μg of S100 was added to individual tubes to start the reaction at 37 °C. The final volume in each reaction was adjusted to 10 μl. All of the reaction was terminated by boiling and analyzed by SDS-PAGE followed by Western transfer and autoradiography. The ubiquitinated IxBo (Ub-IxBo) conjugates were generated by slightly modified Ub-conjugation assays (6, 8), which were essentially the same as the degradation assay except that 1 μg/ml okadacid acid, 1 mM ATP-S, and 100 μM calpain inhibitor I were included in the reaction, and the reactions were carried out for 90 min.
RESULTS

VCP and High Mₗ, Ub-IκBα Are Co-immunoprecipitated in Vivo—IP performed on cell lysates containing active NF-κB detected IκBα, p50, p65, and an unidentified 90-kDa cellular protein (p90) (Refs. 9 and 40; Fig. 1A, top, lanes 1–4). p90 was detected in complexes precipitated by IκBα antisera from various sources and was not detected by the preimmune serum (Ref. 9; Fig. 1C, top, lanes 1 and 6) or the anti-IκBα immune serum preincubated with the immunogenic peptide (40). When the p90-containing IκBα complex was subjected to the in vitro Ub-Pr degradation assay, unlike IκBα molecules, p90 was resistant to degradation (9). To identify p90, IκBα immune complexes were resolved on SDS-polyacrylamide gel, and the p90 band was visualized by Coomassie Blue staining, excised, and subjected to N-terminal peptide sequencing. Although the N terminus of p90 was blocked, peptide sequencing of two of the V8 proteolytic fragments of p90 revealed a total of 20 residues identical to those of the murine valosin-containing protein, VCP (23) (Fig. 1B). IP followed by immunoblotting with either C terminus-specific anti-VCP-1 or N terminus-specific anti-VCP-2, kindly provided by L. Samelson (National Institutes of Health, Bethesda, MD), confirmed that the p90 was VCP (Fig. 1A, bottom). VCP was co-precipitated with IκBα in nondenatured lysate (Fig. 1C, lanes 2), but not in boiled lysate (lanes 7), indicating that VCP physically associates with an IκBα-containing complex. When the reciprocal IP was performed, however, virtually no IκBα was detected in VCP immune complexes (lanes 3, 5, 8, and 10). Although a protein with a similar size to that of IκBα was detected in VCP immune complexes (Fig. 1C, top, lanes 5 and 10), it was not IκBα because it did not react with the antisera specific to the C terminus of IκBα (bottom, lanes 5 and 10). The lack of 36-kDa IκBα in the anti-VCP IP was initially unexpected. However, since VCP is resistant to degradation in Ub-Pr assays (9) and is an ATPase, a common characteristic of molecular chaperones, we hypothesize that VCP is a molecular chaperone that only associates with IκBα molecules that have been modified by ubiquitination. Therefore, the 36-kDa IκBα would not be detected in VCP complexes. Based on this hypothesis, two predictions were made: 1) in addition to 36-kDa IκBα and VCP, anti-IκBα antisera should precipitate high Mₗ, Ub-IκBα, and 2) anti-VCP IP should also detect high Mₗ, Ub-IκBα molecules. With respect to the first prediction, we previously demonstrated that anti-IκBα-1 and -2 detected high Mₗ, Ub-IκBα proteins in a ladderlike pattern (9). To further substantiate the specificity, we subjected the IκBα immune complexes (Fig. 1A, lanes 1–4) to second IPs using various antisera specific to IκBα (lanes 5–8) or to Ub (lane 10). As predicted, both IPs detected abundant high Mₗ, Ub-IκBα.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis analysis was carried out as described previously (44). The highly purified 26 S proteasome isolated from human red blood cells was resolved by two-dimensional isoelectric focusing followed by SDS-gel electrophoresis.

Fig. 1. Identification of VCP and high Mₗ IκBα in IκBα immune complexes. A, DB cells were metabolically labeled with [³⁵S]methionine/cysteine, and the cell lysates were subjected to single IPs (lanes 1–4) or sequential double IPs (lanes 5–10) with the specified antisera. Anti-VCP-4 immune serum was used in the second IP in lane 9. Three times as much lysate were used in each of lanes 5–10 as in lanes 1–4. The immune complexes were separated by SDS-PAGE, transferred onto membrane, and visualized by autoradiography (top; the exposure time for lanes 5–10 is longer than that for lanes 1–4). The membrane was subsequently immunoblotted (IB) with anti-VCP-4 (bottom). The gel mobilities of Mₗ standards (in kDa), VCP, and unmodified IκBα are indicated. The small arrow above the 68-kDa standard indicates the c-Rel protein co-immunoprecipitated with IκBα, and the line above the arrow indicates the 85-kDa protein. B, amino acid identity between the murine VCP sequence and two polypeptides generated from V8 proteolytic fragments of p90 revealed a total of 20 residues identical to those of the murine valosin-containing protein, VCP. The gel was resolved by two-dimensional isoelectric focusing followed by SDS-PAGE, transferred to membranes, and detected by autoradiography. C, DB cells were metabolically labeled with [³⁵S]methionine/cysteine and separated into two parts; one half was lysed in RIPA buffer without SDS (lanes 1–5), and the other half was lysed by boiling for 5 min in RIPA buffer containing 0.5% SDS and then diluted to a final SDS concentration of 0.1%. Aliquots of the lysates were immunoprecipitated with the indicated sera (anti-IκBα-1 was used for IκBα). The washed precipitates were separated by SDS-PAGE, transferred to membranes, and detected by autoradiography (top). The mobilities of the molecular mass standards (in kDa), VCP, and IκBα are indicated. The upper portion of the membrane was subsequently immunoblotted with anti-VCP-2 (middle). The faint bands with molecular mass larger than 90 kDa detected in lanes 6 and 7 are nonspecific. The lower portion of the membrane was immunoblotted with anti-IκBα-1, and the 36-kDa IκBα is indicated (bottom).
molecules but not the c-Rel proteins co-precipitated with IκBα (the 80-kDa band in lanes 1–4, indicated by a small arrow on the left). By contrast, IκBα IP followed by VCP immunoprecipitation did not detect similar high Mr proteins (lane 9). Predominant species with molecular masses of 90 and 85 kDa were noted. Although the majority of p90 precipitated by anti-IκBα was indeed VCP, as shown in the second IP with anti-VCP (lane 9), a minor portion of p90 co-migrating with VCP was Ub-IκBα, because it was reprecipitated by both anti-IκBα (lanes 5–8) and anti-Ub (lane 10) but not reactive to anti-VCP in the subsequent immunoblot (bottom panel).

To test the second prediction, we generated six polyclonal antisera against VCP (Fig. 2A) and examined whether high Mr, Ub-IκBα in VCP immune complexes. A, DB cells were metabolically labeled with [35S]methionine/cysteine and lysed in RIPA buffer containing 0.1% SDS. The cell lysates were immunoprecipitated in the same buffer with the specified preimmune sera (P) or the corresponding anti-VCP immune sera (I). Immune complexes were separated by SDS-PAGE, transferred to membrane, and visualized by autoradiography. B, 35S-labeled DB cell lysates were subjected to single IPs (lanes 1–4) or sequential double IPs (lanes 5–13) with the specified antisera. Three times as much lysates were used in each of lanes 5–13 as in lanes 1–4. After SDS-PAGE, Western transfer, and autoradiography (top; lanes 1–4 are from a 2-day exposure, and lanes 5–13 are from a 20-day exposure), the membrane was immunoblotted (IB) with anti-VCP-3 (bottom).

FIG. 2. Generation of VCP antisera and co-immunoprecipitation of high Mr, Ub-IκBα in VCP immune complexes. A, DB cells were metabolically labeled with [35S]methionine/cysteine and lysed in RIPA buffer containing 0.1% SDS. The cell lysates were immunoprecipitated in the same buffer with the specified preimmune sera (P) or the corresponding anti-VCP immune sera (I). Immune complexes were separated by SDS-PAGE, transferred to membrane, and visualized by autoradiography. B, 35S-labeled DB cell lysates were subjected to single IPs (lanes 1–4) or sequential double IPs (lanes 5–13) with the specified antisera. Three times as much lysates were used in each of lanes 5–13 as in lanes 1–4. After SDS-PAGE, Western transfer, and autoradiography (top; lanes 1–4 are from a 2-day exposure, and lanes 5–13 are from a 20-day exposure), the membrane was immunoblotted (IB) with anti-VCP-3 (bottom).

To further characterize the association of VCP with Ub-IκBα, ultracentrifugation-fractionation analyses were performed (Fig. 3, A and B). Human CA46 lymphoma cells were lysed, and the lysate was separated by 10–40% glycerol density gradient centrifugation and fractionated. The odd-numbered fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-VCP-2 immune serum (Fig. 3A, part a). The majority of VCP (fractions 7–15) migrated similarly to the marker thyroglobulin (data not shown) with an apparent sedimentation coefficient of 19 S, consistent with the previous report (24). Since thyroglobulin has a molecular mass of 650 kDa, it is compatible with the previous findings that VCP and other ATPase family proteins consist of homohexamers, reminiscent of the oligomeric nature of molecular chaperones. When the same fractions were analyzed by blotting with preimmune (part b) or anti-IκBα-1 immune serum (part c), only the high Mr, IκBα and not the unmodified IκBα co-sedimented with VCP in the 19 S fractions (fractions 7–13). As the previous experiments, we also detected a prominent 85-kDa (and less reproducibly, a 90-kDa) anti-IκBα-reactive protein, representing a major species of Ub-IκBα (also see Fig. 3B) in CA46 cells. It was noted that the peak fractions of VCP

serum to VCP, because it was not reactive to anti-VCP in a subsequent immunoblot (bottom panel). Taken together, these results indicate that, in cell lysates, VCP and high Mr, Ub-IκBα are present in the same complexes.

Ub-IκBα Physically Associates and Co-sediments with VCP in the 19 S Fractions, while the Unmodified IκBα Sediments in Fractions Deficient in VCP—To further characterize the association of VCP with Ub-IκBα, ultracentrifugation-fractionation analyses were performed (Fig. 3, A and B). Human CA46 lymphoma cells were lysed, and the lysate was separated by 10–40% glycerol density gradient centrifugation and fractionated. The odd-numbered fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-VCP-2 immune serum (Fig. 3A, part a). The majority of VCP (fractions 7–15) migrated similarly to the marker thyroglobulin (data not shown) with an apparent sedimentation coefficient of 19 S, consistent with the previous report (24). Since thyroglobulin has a molecular mass of 650 kDa, it is compatible with the previous findings that VCP and other ATPase family proteins consist of homohexamers, reminiscent of the oligomeric nature of molecular chaperones. When the same fractions were analyzed by blotting with preimmune (part b) or anti-IκBα-1 immune serum (part c), only the high Mr, IκBα and not the unmodified IκBα co-sedimented with VCP in the 19 S fractions (fractions 7–13). As the previous experiments, we also detected a prominent 85-kDa (and less reproducibly, a 90-kDa) anti-IκBα-reactive protein, representing a major species of Ub-IκBα (also see Fig. 3B) in CA46 cells. It was noted that the peak fractions of VCP
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FIG. 3. Co-sedimentation and physical association of Ub-IκBα with VCP, and no binding of VCP to the NF-κB p50/p65 complexes. CA46 cells were lysed with RIPA buffer without SDS, and the cleared lysate was centrifuged through a 10–40% glycerol gradient. Twenty-six 0.5-ml fractions, with fraction 1 being the bottom and fraction 26 being the top, were collected. A, an aliquot of 40 µg of protein from each odd-numbered fraction was resolved by SDS-PAGE, electrophoretically transferred onto a membrane, and immunoblotted (IB) with anti-VCP-2 (part a). An identical membrane was immunoblotted first with preimmune (part b) and then with immune anti-IκBα-1 serum (panel c). The gel mobilities of molecular size standards (in kDa), high M₆, Ub-IκBα, and the unmodified 36-kDa IκBα are indicated. The open triangles indicate a nonspecific reactivity also detected by the preimmune serum, and the dot to the left of part c indicates the 85-kDa Ub-IκBα. B, equal amounts from fractions 7–13 and 17–20 were combined and named 19 S pool, and these complexes sedimented in the 19 S fractions (Fig. 3 lane 19). In the 4.5 S pool, only high M₆ IκBα, but not 36-kDa IκBα, was detected in the IκBα IP (part b, lane 1), and a significant level of VCP was co-precipitated with high M₆ IκBα (part a, lane 1). The prominent 85-kDa high M₆ IκBα was also reactive to Ub antiserum (part c, lane 1), confirming that it is a Ub-IκBα conjugate. As in Fig. 1, anti-VCP-1 did not co-precipitate detectable amounts of IκBα (part b, lane 2). This was partially a result of competition between the antibody and the IκBα molecules for binding to the same C terminus of VCP (data not shown). In the 4.5 S pool, the majority of IκBα was unmodified (part b, lane 3, marked as IκBα). Although a Ub-IκBα conjugate with a molecular mass slightly smaller than 85 kDa was also detected, no VCP was co-precipitated (part a, lane 3). Actually, these conjugates were present in fractions 15–17, which were deficient in VCP (Fig. 3 lane 1). A low level of VCP was detected in IPs of the 4.5 S pool (part a, lane 4). This probably represents monomeric or dimeric VCP, which does not readily associate with IκBα. In summary, these results indicate that, in vitro, VCP preferentially binds Ub-IκBα over the unmodified 36-kDa IκBα, in agreement with a chaperone role proposed for VCP.

NF-κB p50/p65 Complexes Are Not Associated with VCP—It has been reported that Ub-IκBα remained bound to NF-κB in vitro (8) but dissociated from NF-κB in vivo (14). Thus, it is possible that ubiquitination per se is not sufficient to dissociate...
IκBα from NF-κB complexes, whereas VCP, which is lacking in that specific in vitro assay, may serve as a molecular chaperone that releases Ub-IκBα from NF-κB in vivo. We examined whether the VCP-bound Ub-IκBα conjugates were free from or bound to NF-κB in vivo and whether exogenously added VCP could bind to the purified NF-κB complexes (Fig. 3C). Immune complexes precipitated by various antisera reactive to different regions of p50 and p65 did not contain detectable levels of VCP (one such example was shown in lanes 1 and 4 for p50 and p65, respectively) or Ub-IκBα (data not shown). Further incubation of NF-κB precipitates with VCP did not result in detectable binding of VCP to NF-κB (lanes 2 and 5) or diminution (lanes 3 and 6) of the input free VCP (lane 10). By contrast, IκBα immune complexes contained significant amounts of VCP (lane 7), and almost all of the added VCP could further bind to the complexes (lanes 8–17). These results suggest that VCP-p50/p65 immune complexes do not contain detectable levels of VCP (Fig. 3C) or Ub-IκBα conjugates (Ref. 14; data not shown) in vivo. The NF-κB-Ub-IκBα complex detected in vitro by Chen et al. (8) is probably an unstable intermediate that is readily dissociated by VCP in vivo (see model in Fig. 9).

**Ubiquitinated IκBα Binds to VCP in Vitro**—To further characterize the VCP-IκBα association, we performed in vitro binding assays using GST-IκBα and GST-VCP full-length fusion proteins. VCP (Fig. 4, lane 1) and IκBα (lane 4) were in vitro translated in the presence of [35S]methionine/cysteine in rabbit reticulocyte lysates and incubated with glutathione-Sepharose beads containing either GST or GST fusion proteins. Translated VCP and IκBα bound to GST-IκBα (lane 3) and GST-VCP (lane 7; also see “Discussion”), respectively, but not to the control GST (lanes 2 and 6). PhosphorImager (Molecular Dynamics) scanning showed that about 5–10% of the input VCP was bound to GST-IκBα beads, and 15% of the input IκBα bound to GST-VCP beads. A longer exposure showed a smeary pattern at the high Mr region in lanes 4 and 7 (data not shown), suggesting that high Mr species of IκBα, presumably the Ub-IκBα conjugates, were present in the in vitro translated product and could associate with GST-VCP. The reticulocyte lysate is known to contain enzymes required for ubiquitin conjugation; therefore, it is capable of supporting ubiquitination of IκBα in the in vitro translation reaction. To demonstrate the binding between this high Mr, Ub-IκBα and VCP, we performed Ub conjugation reactions (8, 16) on the translated IκBα to increase the yield of Ub-IκBα conjugates (lane 5) and then used the products in binding assays (lanes 8 and 9). Similar to the long exposure of lane 7, a smeary pattern above and including IκBα was detected in VCP-bound IκBα (lane 9), and scanning analysis showed an approximately 40% binding efficiency, significantly higher than that of the non-Ub-enriched preparations. Subsequent anti-IκBα (lanes 10–13) and anti-Ub (lanes 14–17) immunoblotting confirmed that these high Mr proteins were Ub-IκBα conjugates. The dissimilar patterns observed in lanes 13 and 17 could result from the difference in the number of epitopes recognized by different antibodies. The high Mr Ub-IκBα molecules have many more Ub-specific epitopes than IκBα-specific epitopes and therefore should have a stronger anti-Ub reactivity. More importantly, VCP probably also bound to ubiquitinated proteins other than Ub-IκBα in the assay mixture, resulting in a higher reactivity in lane 17 than in lane 13. The immunoreactivity was specific to Ub-IκBα, because GST-VCP alone was not reactive to either antiserum (data not shown). Furthermore, when B cell lysates were used in the binding assays (lanes 18–21), abundant high Mr Ub-IκBα conjugates also specifically bound to VCP (lanes 19 and 21). Notably, among the VCP bound Ub-IκBα proteins, a major 85-kDa species was again detected in cellular lysates and in in vitro translated IκBα (marked by dots in lanes 13, 17, 19, and 21; also see Figs. 1–3), representing a prominent species of Ub-IκBα that bound to VCP.

**Phosphorylation and Ubiquitination of IκBα Are Critical for VCP Association, and both N- and C-terminal Domains of IκBα Are Required for IκBα Degradation**—The N-terminal serines 32 and 36 (7, 10, 11, 15) and lysines 21 and 22 (12, 13, 15) have been shown to be the major signal-induced phosphorylation and ubiquitination sites, respectively. To demonstrate the physiological significance of VCP in the IκBα degradation pathway, we studied how IκBα mutations that blocked the upstream events, specifically phosphorylation and ubiquitination, affected the VCP association (Fig. 5A) and the subsequent degradation (Fig. 5B; summarized in Fig. 5C). We subjected wild-type and mutant IκBα to Ub conjugation reactions (Fig. 5A, lanes 2–7) before performing VCP binding assays (lanes 8–21). In agreement with the previous reports, the constructs with intact phosphorylation and ubiquitination sites were further ubiquitinated (lanes 2 and 5), and no significant ubiquitination was detected in IκBα with mutated phosphorylation sites (S32A/S36A, lane 6), mutated ubiquitination sites (R21R/K22R, lane 7), or IκBα lacking both sites (37–317, lane 4). Interestingly, the mutant IκBα (23–317), although missing the major ubiquitination sites Lys22 and Lys22, was detectably ubiquitinated (lane 3). This result agrees with a similar finding.
by DiDonato et al. (15) and suggests that other Lys residues in the molecule could accept Ub. The subsequent binding experiments revealed that all of the constructs capable of ubiquitination could bind to VCP (lanes 9, 11, 13, and 17), the ubiquitination-defective mutants all failed to bind VCP (lanes 15, 19, and 21). Taken together, the data indicate that phosphorylation and ubiquitination of IκBα are critical for VCP association, and IκBα ubiquitination is necessary for VCP binding (see Fig. 5C).

Using the in vitro Ub-Pr degradation assays (9), we further examined how these IκBα mutants affected the ultimate degradation (Fig. 5B). In this assay, in vitro translated [35S]cysteine-labeled IκBα was used as the substrate, 50 μg of protein from the S100 fraction extracted from B cells was used as the enzyme source, and the disappearance/degradation of the substrate was assayed. The wild-type IκBα was readily degraded.

Fig. 5. Requirement of IκBα phosphorylation and ubiquitination for VCP association and requirement of both N- and C-terminal domains of IκBα for degradation. A, in vitro translated, [35S]-labeled wild-type (lane 1) and various mutants of IκBα were subjected to Ub conjugation reactions (lanes 2–7). The reaction products were used in the binding assays with beads containing either GST control (even-numbered lanes from lane 8 to lane 21), or GST-VCP (odd-numbered lanes from lane 8 to lane 21). The samples were analyzed by SDS-PAGE, transferred onto membrane, and visualized by autoradiography. The closed circle, the open circle, and the arrowhead indicate the translation products of constructs 23–317, 37–317, and 1–242, respectively. The faint bands indicated by open triangles are nonspecific reactivities that are present in most reactions. B, in vitro translated, [35S]-labeled wild-type (lanes 1–4) and deletion mutants (lanes 5–8 for mutant 23–317; lanes 9–12 for mutant 37–317, and lanes 13–16 for combination) of IκBα were used in the in vitro Ub-Pr degradation assay. The discrete bands represent the undegraded substrates remained in the reactions at the indicated time points. C, the results from in vitro ubiquitination, VCP-binding, and degradation assays were summarized. The data on phosphorylation were based on published reports (Refs. 7–15; see Introduction).
that VCP binding is necessary but not sufficient for IkB degradation. To further demonstrate the functional involvement of VCP in IkB degradation at a biochemical level, we correlated the proteolytic activity (Fig. 6A) contained in purified lysates. Pr

fractions were isolated from the cytosolic S100 fraction of the human B cell line, and an equal amount of protein from each fraction was resolved by SDS-PAGE and analyzed by immunoblotting. Preimmune serum showed no reactivity (Fig. 6B), whereas anti-VCP-2 immune serum clearly detected VCP in Pr

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(lanes 2), and higher in the S100 fraction (lanes 3) than in the crude cytosol (lanes 4).

VCP Antibodies Deplete Proteasome Activity. While Adding Back Immunopurified VCP Complexes Restores the Proteolytic Activity—We further determined if depletion of VCP (Fig. 6D) resulted in a loss of proteasome activity (Fig. 6E). VCP-containing complexes were removed from the S100 fraction by subjecting it to repeated cycles of anti-VCP-1 IPs. After the third IP, virtually no VCP could be further precipitated (Fig. 6D, lane 5). When the VCP-depleted lysate was used as the enzyme source in the in vitro degradation assay, no IkB-degrading activity was detected, as shown by the undiminished level of substrate that remained at the end of the assay (Fig. 6E, lane 5). In contrast, when the VCP complexes obtained from each of the first three IP cycles were added back to the VCP-depleted lysate, the IkB-degrading activity was restored, as shown by the loss of the input substrate at the end of the assay (Fig. 6E, lanes 2–4). These results demonstrate that VCP is necessary for IkB degradation and can be a functional component of the Ub-Pr pathway involved in IkB degradation.

VCP Co-migrates with Subunit 2 of the Proteasome in Biochemically Purified 26 S Proteasome and Is Co-immunoprecipitated with the 26 S Proteasome—The 26 S proteasome can be resolved into many subunits by SDS-PAGE (Refs. 31, 32, and 48; Fig. 7B, part a; Fig. 8A, lane 6). Based on the observation that the molecular mass of VCP is similar to that of S2 of the purified 26 S proteasome, we determined the presence of VCP in 26 S proteasome preparations (Fig. 7). Partially purified (Fig. 7A, lanes 1 and 5) and highly purified (lanes 2 and 6) 26 S proteasomes isolated from human red blood cells (46) were compared with the Pr

fraction isolated from a human B cell (Fig. 5B, lanes 1–4), indicating that the assay system contained all of the needed ingredients to facilitate the degradation. Compatible with the model, the mutants that could not bind VCP all failed to be degraded (mutant 37–317 is shown in lanes 5–8 and 13–16; other mutants are summarized in Fig. 5C). However, mutants that could bind VCP were not necessarily degraded (mutant 1–242 is shown in lanes 9–16). These results suggest that VCP binding is necessary but not sufficient for IkB degradation. The failure in degradation is not a result of the lack of needed reagents in the in vitro assay but a result from the mutations in the substrates themselves. Consistent with a previous report (47) that both N- and C-terminal domains of IkBα were required for degradation, we also found that both N- (mutant 23–317) and C- (mutant 1–242) terminally truncated mutants, although capable of VCP binding, were resistant to degradation. The assays also showed that the required N- and C-terminal domains have to be on the same molecule, because incubating the two mutants together did not restore the degradation (lanes 13–16).

The Level of VCP Correlates with the Proteolytic Activity in the Ub-Pr Assay—To further demonstrate the functional involvement of VCP in IkBα degradation at a biochemical level, we correlated the proteolytic activity (Fig. 6A) contained in purified lysates. Pr

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and the proteasome-enriched Pr* fraction (lanes 3 and 7) was separated by SDS-PAGE and stained with Coomassie Blue (lanes 1–4; lane 4 shows the mobilities of the size standards of 68 and 43 kDa) or immunoblotted with anti-VCP-2 (lanes 5–7). Aliquots of 5 µg of Pr* were analyzed by immunoblotting with preimmune serum (lane 8), anti-VCP-3 (lane 9), or anti-VCP-1 (lane 10). B, the highly purified 26 S proteasome (as 26 S-1 in A) isolated from human red blood cells was analyzed by two-dimensional isoelectric focusing and SDS-PAGE and transferred onto membrane. The membrane was stained by Ponceau S. (panel A) and then immunoblotted with anti-VCP-2 (part b). The sample on the left side of the gel is a one-dimensional analysis of the highly purified proteasome, and the assignments of individual subunits are indicated on the left. The stained VCP and the anti-VCP reactivity are indicated by arrows.

FIG. 7. Co-purification of VCP with the 26 S proteasome. A, lane 1 and 5), the highly purified 26 S proteasome (26 S-1) (lanes 2 and 6), and the proteasome-enriched Pr* fraction (lanes 3 and 7) was separated by SDS-PAGE and stained with Coomassie Blue (lanes 1–4; lane 4 shows the mobilities of the size standards of 68 and 43 kDa) or immunoblotted with anti-VCP-2 (lanes 5–7). Aliquots of 5 µg of Pr* were analyzed by immunoblotting with preimmune serum (lane 8), anti-VCP-3 (lane 9), or anti-VCP-1 (lane 10). B, the highly purified 26 S proteasome (as 26 S-1 in A) isolated from human red blood cells was analyzed by two-dimensional isoelectric focusing and SDS-PAGE and transferred onto membrane. The membrane was stained by Ponceau S (part a) and then immunoblotted with anti-VCP-2 (part b). The sample on the left side of the gel is a one-dimensional analysis of the highly purified proteasome, and the assignments of individual subunits are indicated on the left. The stained VCP and the anti-VCP reactivity are indicated by arrows.

S2, highly purified 26 S proteasomes (as in Fig. 7A, lane 2) from human red blood cells were subjected to two-dimensional gel electrophoresis analysis (Fig. 7B). The stained filter showed a major protein with a pI around 5.1 (marked by an arrow in part a) and a more basic triplet that co-migrated to the S2 position; immunoblotting confirmed that VCP was the more acidic species (marked by an arrow in part b). The PI values of S2, cellular VCP, and in vitro translated VCP were all estimated to be between 5 and 6 (data not shown), consistent with the reported pI of 5.5 for the Xenopus counterpart, p97 (49).

We further examined whether VCP was co-immunoprecipitated with the 26 S proteasome (Fig. 8). Labeled cell lysates were immunoprecipitated with antisera specific to VCP (lanes 1–3), subunit 4 (S4, lane 4), subunit 5 (S5, lane 5), or the 26 S proteasome (lane 6). Although the oligopeptide-specific anti-VCP antisera did not readily precipitate recognizable proteasome subunits (Fig. 8A, compare lanes 1 and 2 with lanes 4–6), anti-VCP-3, raised against GST-VCP full-length fusion protein, co-precipitated proteins of sizes similar to those of subunits 1–11 (lane 3) (32). Subsequent immunoblot (Fig. 8C) and immunoprecipitation (data not shown) analyses further confirmed the co-precipitation of VCP and S4. Correspondingly, IPs using antisera specific to S4, S5, and the 26 S proteasome all detected small amounts of VCP (Fig. 8, A and B, lanes 4–6).

In conclusion, VCP is co-purified with 26 S proteasome biochemically and immunologically.
In this model, two non-mutually exclusive roles, as a molecular chaperone and/or as a physical component of the 26 S proteasome, are proposed for VCP. The hypothesis of VCP being a molecular chaperone was first suggested by our observation that when the p90(VCP)-containing I\(\kappa\)B\(\alpha\) complexes were subjected to the

\textit{in vitro} degradation assay, only I\(\kappa\)B\(\alpha\) and not p90(VCP) was degraded (9). This is consistent with the nature of a molecular chaperone, which should be recycled and not consumed after the degradation of the substrates. In addition, VCP is an ATPase and forms an oligomeric ring structure (24, 49); both are common properties of molecular chaperones. One important characteristic of a molecular chaperone is its ready association with specific substrates. If VCP is truly a molecular chaperone in Ub-Pr-mediated degradation of I\(\kappa\)B\(\alpha\), it should preferentially bind the Ub-tagged I\(\kappa\)B\(\alpha\). The \textit{in vivo} IP and co-sedimentation experiments clearly showed such a substrate specificity, in that 1) I\(\kappa\)B\(\alpha\) IP detected physically associated VCP and high M\(_2\) Ub-I\(\kappa\)B\(\alpha\) in addition to the 36-kDa I\(\kappa\)B\(\alpha\); 2) serial IPs using VCP followed by I\(\kappa\)B\(\alpha\) antisera showed that VCP immune complexes contained high M\(_2\) Ub-I\(\kappa\)B\(\alpha\) but not 36 kDa-I\(\kappa\)B\(\alpha\); 3) VCP and the high M\(_2\) Ub-I\(\kappa\)B\(\alpha\) co-sedimented in the 19 S fractions; 4) 36-kDa I\(\kappa\)B\(\alpha\) sedimated in the 4.5 S fractions lacking VCP; 5) in the 19 S pool, VCP and Ub-I\(\kappa\)B\(\alpha\) were co-precipitated in the same complex; and 6) in the 4.5 S pool, the majority of I\(\kappa\)B\(\alpha\) was not ubiquitinated and was free from VCP. The \textit{in vitro} binding experiments also showed a ready association of VCP with Ub-I\(\kappa\)B\(\alpha\). When cell lysates were used as the source of I\(\kappa\)B\(\alpha\), only Ub-I\(\kappa\)B\(\alpha\) and not the 36-kDa I\(\kappa\)B\(\alpha\) bound to VCP, consistent with the \textit{in vivo} analysis. Furthermore, when \textit{in vitro} translated I\(\kappa\)B\(\alpha\) constructs with various mutations were used in the assays (Fig. 5, A and C), a perfect correlation between I\(\kappa\)B\(\alpha\) ubiquitination and VCP binding was detected; all I\(\kappa\)B\(\alpha\) variants capable of ubiquitination could bind VCP, and all constructs defective in ubiquitination could not. It was noted that when, and only when, the \textit{in vitro} translated I\(\kappa\)B\(\alpha\) was used in the \textit{in vitro} binding assays, in addition to Ub-I\(\kappa\)B\(\alpha\), the 36-kDa I\(\kappa\)B\(\alpha\) was also detected in VCP complexes (Figs. 4 and 5A). It is presently unclear whether the 36-kDa I\(\kappa\)B\(\alpha\) genuinely binds to VCP \textit{in vitro} or is detected as a consequence of rapid deubiquitination under the specific experimental condition. The recent identification of a Ub isopeptidase, a component of the 26 S proteasome, that quickly removes Ub chains, commonly found in \textit{in vitro} conditions, by this isopeptidase is consistent with our findings. It is also formally possible that this 36-kDa I\(\kappa\)B\(\alpha\) is hyperphosphorylated but not ubiquitinated. Although not detected \textit{in vivo}, this form of I\(\kappa\)B\(\alpha\) may bind to VCP \textit{in vitro}. Interestingly, we frequently detected binding of VCP to an 85-kDa Ub-I\(\kappa\)B\(\alpha\) in cell lysates (Figs. 1–4) and in Ub-I\(\kappa\)B\(\alpha\) generated from the Ub conjugation reaction (Fig. 4). In addition, we also detected prominent Ub-I\(\kappa\)B\(\alpha\) species with M\(_2\) of 120 and 90 kDa (Figs. 1A, 2B, and 4). Consistently, Ub-I\(\kappa\)B\(\alpha\) species with similar sizes were also detected in other studies (8, 14, 15). These Ub-I\(\kappa\)B\(\alpha\) molecules probably represent major polyubiquitinated species that are preferentially bound by VCP. In summary, VCP readily binds Ub-I\(\kappa\)B\(\alpha\) conjugates, requires I\(\kappa\)B\(\alpha\) ubiquitination for binding, and clearly demonstrates a binding preference for them over the unmodified I\(\kappa\)B\(\alpha\) \textit{in vivo}, consistent with the property of a molecular chaperone.

Chen et al. (8) showed that Ub-I\(\kappa\)B\(\alpha\) was still bound to NF-\(\kappa\)B \textit{in vitro}, while Roff et al. (14) showed that Ub-I\(\kappa\)B\(\alpha\) was dissociated from p50-containing NF-\(\kappa\)B \textit{in vivo}. The discrepancy between the two findings suggests the existence of an \textit{in vivo}-specific molecular chaperone that displaces the NF-\(\kappa\)B from Ub-I\(\kappa\)B\(\alpha\). We identified VCP as the candidate chaperone to fulfill this function. Our data demonstrating VCP binding to I\(\kappa\)B\(\alpha\) but not p50-p65 complexes is consistent with the model...
that following IκBα ubiquitination, VCP binds the Ub-IκBα conjugates and releases NF-κB to allow for its nuclear translocation. It is noted that, contradictory to the findings of Roff et al. (14), DiDonato et al. (15) reported that Ub-IκBα remained bound to the p65-containing complexes in cells treated with proteasome inhibitor. At present, we do not have a definitive explanation for this discrepancy. However, both studies were performed in proteasome inhibitor-treated cells, in which unusually high amounts of Ub-IκBα could be accumulated. It is conceivable that when the level of Ub-IκBα exceeds the level of available VCP, presumably as in the latter but not in the former study, Ub-IκBα-p65 complexes can be detected.

To demonstrate the involvement of VCP in the signaling pathway of IκBα degradation, both mutant and biochemical studies were performed. We examined how the phosphorylation-and/or ubiquitination-defective IκBα mutants affected the VCP binding and the ultimate degradation (summarized in Fig. 5C). A perfect correlation between ubiquitination and VCP binding was detected, supporting the model that VCP binding requires the upstream ubiquitination. Interestingly, the mutant 37–317, lacking both phosphorylation and ubiquitination sites, was defective in VCP binding; whereas the mutant 23–317, lacking the major ubiquitination sites, was capable of ubiquitin conjugation and VCP binding. This result suggested two points. First, other lysine residues in the IκBα molecule could also accept Ub, which then signaled for VCP binding. This notion is consistent with another report (15) and is supported by studies reporting that the Ub-conjugating apparatus has the capacity to utilize alternative lysines when the primary acceptors are disrupted (51, 52). However, if alternative Lys residues can accept Ub molecules, it is unclear why the mutant K21R/K22R was defective in ubiquitination and VCP binding, although consistent with other reports. Second, the recognition of Ub by VCP is probably not highly site-specific. Ub attached to Lys residues other than the major sites Lys<sup>31</sup> and Lys<sup>32</sup> can also be efficiently recognized by VCP, at least in our in vitro binding assays. Presently, we do not know how VCP recognizes Ub-IκBα conjugates and whether VCP can recognize Ub chains attached to all of the nine Lys residues in IκBα. Nevertheless, this finding further substantiated the requirement of ubiquitination for VCP binding.

The in vitro degradation assays showed that not only were the mutants defective in VCP binding unable to be degraded, but several mutants capable of binding VCP were also resistant to degradation (Fig. 5C). These results support the model that while binding with VCP is necessary for degradation, it is not sufficient. In agreement with a previous study (47), we also found that both N- and C-terminal domains of IκBα were required for degradation. This is consistent with the findings that the C-terminal acidic PEST (Pro-Glu-Ser-Thr) sequences are often associated with rapid protein turnover and frequently found in Ub-Pr substrates. Moreover, the N- and C-terminal domains must be present in a cis relationship, suggesting that a successful degradation requires the presence of a complete protein domain and that intramolecular conformation of the IκBα protein may be important in the process.

All of our data are compatible with both roles proposed for VCP, one being a molecular chaperone with a distinct hexameric structure and the other being a component of the 26 S proteasome. Biochemically, VCP co-purifies with the 26 S proteasome; immunologically, VCP co-immunoprecipitates with subunits of the proteasome, all supporting the “component” role. Interestingly, the VCP antisera raised against synthetic oligopeptides co-precipitated the high Mr Ub-IκBα but not the proteasome components. On the other hand, anti-VCP-3, which was raised against a full-length VCP fusion protein, readily co-precipitated the proteasome subunits, but only co-precipitated low levels of Ub-IκBα. These data along with the serial IP experiments (data not shown) seem to suggest that there are at least two populations of VCP in the cell; while the peptide-specific VCP antisera may recognize the proteasome-free VCP, anti-VCP-3 may react more readily with the proteasome-bound VCP. It is conceivable that VCP may exist in a state of equilibrium between a mobile form, which presumably functions as a molecular chaperone, and a relatively immobile form that assembles on the top of the 26 S proteasome. Functionally, VCP may hydrolyze ATP (24) to provide the energy needed for the unfolding of the modified IκBα molecule and its entry into the 20 S proteolytic core. Analogous dual activities have been demonstrated in VCP relatives, ClpA and ClpX in *Escherichia coli*. Both proteins appear to exhibit intrinsic ATP-dependent chaperone activities (53) and are supposed to be part of the regulatory structure on top of the cylinder-shaped Clp protease (54). These notions also bear similarities with a recent report that Ub chain-binding protein Mcb1, subunit 5a, exists in two populations, 26 S proteasome-bound and -free forms (55).

Although VCP co-purifies with the 26 S proteasome and co-migrates with S2 of the 19 S cap complex on SDS-gels, it is not identical to S2. Recently, an unrelated protein named TRAP-2 (56), corresponding to the more basic triplet of S2 on our two-dimensional gel (Fig. 7B), has been identified as S2 by protein sequencing. It is possible that there is more than one protein constituting S2. This biochemical heterogeneity observed in a protein resolved by SDS-PAGE to apparent homogeneity is preceded by the finding that S5 of the 26 S proteasome consists of two unrelated proteins, S5a and S5b (43, 44). Alternatively, the regulatory 19 S cap may not be a single homogeneous entity, and the VCP complex may represent an alternative version of the 19 S cap that confers specificity to certain substrates such as IκBα. Thus, subunit composition and the functional capability of the 26 S proteasome may vary with physiological conditions.

VCP has been highly conserved during evolution and found in every tissue examined to date. The paradox present in the high sequence homology and the variety of functions suggests a basic and critical role for VCP/Cdc48p/p97. Recently, cell cycle progression, cytokine-induced signal transduction pathways, and endoplasmic reticulum degradation were reported to be regulated by the Ub-Pr pathways (16–30, 57, 58). By identifying the involvement of VCP in IκBα degradation through the Ub-Pr pathway, we may provide a potential link between the 26 S proteasome and other Ub-Pr substrates, thus offering an explanation for the paradox. In conjunction with our findings, it would be interesting to examine whether VCP is also involved in other Ub-Pr-mediated processes in the context of proteasomes.

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Involvement of Valosin-containing Protein, an ATPase Co-purified with IκBα and 26 S Proteasome, in Ubiquitin-Proteasome-mediated Degradation of IκBα

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