

Sequestosome 1/p62 Is a Polyubiquitin Chain Binding Protein Involved in Ubiquitin Proteasome Degradation

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Herein, we demonstrate that the ubiquitin-associated (UBA) domain of sequestosome 1/p62 displays a preference for binding K63-polyubiquitinated substrates. Furthermore, the UBA domain of p62 was necessary for aggregate sequestration and cell survival. However, the inhibition of proteasome function compromised survival in cells with aggregates. Mutational analysis of the UBA domain reveals that the conserved hydrophobic patch MGF as well as the conserved leucine in helix 2 are necessary for binding polyubiquitinated proteins and for sequestration-aggregate formation. We report that p62 interacts with the proteasome by pull-down assay, coimmunoprecipitation, and colocalization. Depletion of p62 levels results in an inhibition of ubiquitin proteasome-mediated degradation and an accumulation of ubiquitinated proteins. Altogether, our results support the hypothesis that p62 may act as a critical ubiquitin chain-targeting factor that shuttles substrates for proteasomal degradation.

Sequestosome 1/p62 is a cellular protein which was initially identified as a phosphotyrosine-independent ligand of the Src homology 2 (SH2) domain of p56^{lck} (27). The protein has been cloned by two independent groups as a counteracting protein of the atypical protein kinase C ζ (aPKC ζ) and is also named ZIP for PKC ζ -interacting protein (47, 50). p62 has been shown to bind ubiquitin noncovalently (56), and its overexpression results in large cytoplasmic aggregates (47). We have recently determined that p62 possesses sequence homology with other proteins possessing a ubiquitin-associated (UBA) domain at their C terminus, amino acids 386 to 434 (16), in p62. Interestingly, p62 contains several structural motifs which suggest that it might participate in the formation of multimeric signaling complexes. These domains include an acidic interaction domain (AID/ORCA/PC/PB1) that binds the aPKC, a ZZ finger, a binding site for the RING finger protein TRAF6, two PEST sequences, and the UBA domain (16).

Ubiquitin is a small polypeptide of 76 amino acids that can be covalently attached to other proteins. Monoubiquitination serves as a novel endocytosis signal (19), whereas polyubiquitin chains target substrates for degradation by the proteasome (45). p62 has been shown to interact in a noncovalent fashion with polyubiquitin chains (5, 53), which is consistent with reports demonstrating that proteins possessing UBA domains are more likely to bind polyubiquitin chains over monoubiquitin (59). Conjugation of ubiquitin to substrate proteins requires three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Initially, E1 activates ubiquitin by forming a high-energy thioester intermediate with the C-terminal glycine using ATP. The activated ubiquitin is sequentially transferred to E2 and then to E3 which catalyzes isopeptide bond formation between

the activated C-terminal glycine of ubiquitin and an ϵ -amino group of a lysine residue in the substrate (45). However, only HECT-type E3s can form thioester bonds with ubiquitin, while RING E3s such as TRAF6 (23) do not. Consequently, the isopeptide bond that is formed between ubiquitin and the substrate could be formed by either E2 or E3. Following the linkage of the first ubiquitin, additional molecules of ubiquitin are attached to the previously conjugated moiety to form branched polyubiquitin chains employing lysine (K) linkage K29, K48, or K63 (45). It has been proposed that the fate of a substrate may depend on the length of the chain as well as the lysine linkage (K29, K48, or K63) involved in forming the chain. Proteins which possess K48 chains target proteins to the proteasome (45), whereas ubiquitin chains composed of K63 have been shown to possess a role aside from that of proteasomal targeting (38). Ubiquitin itself can be modified at all seven lysine residues, suggesting that chains of K6, K11, K27, and K33, as well as those of K29, K48, and K63, may be found *in vivo*, thereby enhancing the diversity of polyubiquitin chains (44). BLAST analysis of the human genome reveals that there are 26 proteins with UBA domains (J. Pridgeon and M. W. Wooten, unpublished data). Thus, a recognition code is likely needed by each polyubiquitin-binding protein for the efficient sorting of chains of different types. One mechanism involved in coding proteins for sorting may be the ability of each chain to adopt a specific conformation. In this regard, chains of K29, K48, or K63 have been reported to adopt a distinct conformation (57, 58). Therefore, each type of polyubiquitin chain may possess a distinct cellular function and interact with a defined polyubiquitin-binding protein through the ability of a UBA domain to recognize a particular type of chain.

Insoluble ubiquitin-protein aggregates are tightly linked to neuronal degeneration and are believed to be a common feature of several age-related neurodegenerative diseases (1). However, the precise molecular mechanism which contributes to aggregate formation and compromised cell survival is not

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clear. Sequestosome 1/p62 has been colocalized to ubiquitin-tau-containing inclusions in the hippocampus and neocortex of patients with Alzheimer's disease but is sparse or absent from healthy age-matched brains free from tangles (31, 32). Thus, this study was undertaken to examine the ability of p62 to bind specific polyubiquitin chains and to establish the role of the UBA domain of p62 in aggregate formation and the relationship of aggregates to cell survival. We observe that the UBA domain of p62 displays a preference for interacting with substrates possessing K63-linked polyubiquitin chains and that, in proteasome-impaired conditions, aggregates compromise cell survival.

MATERIALS AND METHODS

Cell culture and reagents. Cultures of human embryonic kidney 293 (HEK) cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. Subconfluent cultures were transfected by using a mammalian cell transfection kit from Specialty Media, Phillipsburg, Pa. The UBA domain peptide coupled to agarose, GST-S5a agarose, and proteasomal antibodies were obtained from Affiniti Research Products. Monoclonal anti-ubiquitin and antihemagglutinin (anti-HA) as well as polyclonal anti-myc, anti-HA, and anti-TRAF6 antibodies were from Santa Cruz Biotechnology, La Jolla, Calif. Monoclonal antibody to glutathione *S*-transferase (GST) was from Sigma, St. Louis, Mo. The HA-tagged ubiquitin K/R mutants were a generous gift from Cam Patterson, University of North Carolina at Chapel Hill, Chapel Hill, N.C. TRAF6 peptide was synthesized by Alpha Diagnostics, San Antonio, Tex. All other reagents were from Sigma unless otherwise specified.

Immunoprecipitation. HEK 293 cells were transfected with indicated tagged constructs in each experiment employing calcium phosphate transfection (Specialty Media). Alternatively, for confocal microscopy experiments, the cells were transfected with Lipofectamine 2000. After 36 h of transfection, medium was removed from attached cells and plates were gently washed with cold phosphate-buffered saline (PBS). For polyubiquitin interaction *in vivo*, myc-tagged p62 or myc-tagged p62 Δ UBA and HA-tagged ubiquitin were transfected into HEK cells. The cells were lysed for 20 min at 4°C in either pNAS buffer 50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40 [to detect noncovalent interaction] or 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS; used to detect covalent interaction), both containing protease inhibitors. Anti-myc antibody was added to the lysate (750 μ g) for 3 h at 4°C and captured by the addition of mouse immunoglobulin G (IgG) agarose for an additional 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer followed by the addition of SDS sample buffer. The bound proteins were separated on SDS-polyacrylamide gels and subjected to immunoblotting with polyclonal antibody to HA. Detection was performed with ECL reagents (Amersham Biosciences Corp.).

Immunofluorescence and imaging. HEK cells were transfected with Lipofectamine (Gibco) according to the manufacturer's instructions by using 1 μ g of DNA per well. All steps were done at room temperature unless otherwise specified. Cells were washed 26 to 36 h after transfection with PBS. Those cells transfected with green fluorescent protein (GFP)-p62 were fixed in 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) for 10 min. Cells transfected with myc-p62 with or without HA ubiquitin were fixed in 3% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS for 15 min. Cells were then blocked in blocking buffer (3% nonfat dry milk in PBS) for 1 h and incubated with primary antibody, myc tag or HA tag (Santa Cruz), overnight at 4°C. Cells were then washed and incubated with secondary antibody coupled to Texas Red or Oregon Green (Molecular Probes, Portland, Oreg.) for 1 h. Alternatively, to detect proteins colocalized with p62 to the aggregates, staining was performed with TRAF6 (Santa Cruz) as the primary antibody and Texas Red-coupled secondary antibody in the same manner as described above. Coverslips were mounted on slides by using Vectashield (Vector Laboratories Inc., Burlingame, Calif.), and cells were analyzed and imaged with a 100 \times oil immersion objective on a Bio-Rad MRC 1024 confocal microscope. *z* stacks were collected and manipulated with Confocal Assistant (version 4.02; Bio-Rad) and processed by using Adobe Photoshop 6.0 software.

Cell survival assays. HEK 293 cells (in a 24-well plate) were transfected with 1 μ g of myc-tagged wild-type p62 or its mutants Δ UBA and Δ N-term along with 1 μ g of HA-tagged ubiquitin constructs. Twenty-four hours posttransfection, the cells were washed five times with serum-free medium followed by an addition of

N-acetyl-Leu-Leu-norleucine-al (ALLN; 50 μ M) or not for 30 h. Cell survival (39) was assessed by the addition of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega] for 2 h. MTS is bioreduced into a water-soluble formazan product by a dehydrogenase enzyme found in metabolically active cells and is directly proportional to the number of living cells in culture (39). The quantity of formazan product was determined by absorbance readings at 490 nm (Dynatech microplate reader). Alternatively, cells were transfected with GFP-p62, treated or not treated with ALLN, and stained as outlined with a Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, Oreg.). Cells with one or more GFP-p62 aggregates were scored as aggregate positive, whereas cells lacking GFP aggregates were scored as negative. Green cells were scored as alive and red cells were scored as dead. Three random populations of cells on the coverslips (with or without ALLN) with or without aggregates were scored as either dead or alive. The percentage of the population in each group was calculated as a function of the total cells counted (~200).

GST-UBA polyubiquitin interaction. Using the full-length myc-tagged p62 construct as the template, single point mutations at critical conserved amino acids within the UBA domain were generated by Genemed Synthesis, Inc. (San Francisco, Calif.), changing each residue to valine. The mutation of each construct was verified by sequence analysis. To generate a GST-UBA domain construct for each mutant, the UBA domain was excised from the wild-type p62 pcDNA3.1 vector. A BamHI restriction site was engineered 38 bases upstream of the UBA domain in each construct by PCR while an EcoRI cloning site in pcDNA3.1 was utilized downstream. Primers encompassing these two restriction sites were used to amplify the UBA domain of each mutant followed by restriction digestion with BamHI and EcoRI enzymes. The digest was separated by electrophoresis employing low-melting-point agarose-Tris-borate-EDTA. The fragment was gel purified by using a QiaQuick gel extraction kit (QIAGEN) and subcloned into the pGEX-6P1 GST expression vector (Amersham). GST-tagged UBA domain mutant constructs were transformed into JM109 *Escherichia coli* cells. Following induction of cells with IPTG (isopropyl- β -D-thiogalactopyranoside), the cells were lysed in NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, 2 μ g of aprotinin/ml, 2 μ g of leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The GST-tagged UBA domain was bound to glutathione agarose overnight at 4°C followed by washing five times with NETN buffer. The integrity and purity of the preparation were validated by SDS-12% polyacrylamide gel electrophoresis (PAGE) and/or Western blot analysis with GST antibody. GST-bound UBA domains were subsequently used in polyubiquitin chain binding assays. HEK cells were transfected with 12 μ g of HA-tagged wild-type ubiquitin or K/R mutants by the calcium phosphate method. After 36 h, the cells were lysed with binding buffer (20 mM Tris-HCl [pH 7.6], 50 mM NaCl, 0.1% Nonidet P-40, 0.5 M dithiothreitol, 1 mM PMSF). The GST-UBA domain of the p62 protein on beads was washed three times with binding buffer prior to use in an interaction assay. Five micrograms of the GST-UBA domain was added to 750 μ g of lysate and rotated for 2 h at room temperature. The beads were washed three times with binding buffer, and SDS-PAGE sample buffer was added and analyzed by SDS-7.5% PAGE followed by immunoblotting with ubiquitin antibody.

Circular dichroism measurements. Bacteria expressing GST-tagged UBA domains were grown and induced, and GST-tagged UBA domain proteins were purified as described above. The purified UBA domains, wild type or mutant, were isolated by cleavage from the bound GST tag by using PreScission protease (Amersham) according to manufacturer's instructions. Briefly, once the identities of GST-tagged UBA domains were verified by Western blot with GST antibody, bound proteins were washed three times with PreScission protease buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and resuspended to 50% in the same buffer. PreScission protease was added to a concentration of 1 U per 100 μ g of bound protein, and incubation proceeded overnight at 4°C with agitation. The beads were pelleted, and the supernatant was dialyzed overnight at 4°C against NaPO₄ buffer at pH 8.0 in Slide-A-Lyzer dialysis cassettes (Pierce). Following dialysis, samples were concentrated by using Centricon YM-3 centrifugal concentrators (Millipore) to a volume of approximately 200 μ l. Purified peptides (30 to 45 μ M in 33 mM NaPO₄ buffer, pH 8.0) were used to collect circular dichroism (CD) spectra by using an Aviv 62DS spectrometer. All measurements were taken at ambient temperature (~27°C) with a 1-mm-path-length cell. The spectra were collected in a single scan from 240 to 190 nm, with a step size of 0.5 nm and 8 s of average time. CD data were analyzed by using the CONTIN/LL (46) and CDSSTR (25) methods in the CDPro software package. A 43-protein reference set was chosen for calculating the secondary structural elements of the peptides.

Interaction of p62 with the proteasome. To examine p62 interaction with the proteasome, S5a agarose beads (Affiniti) or highly purified proteasomes (Af-

finiti) were used in pull-down assays (55). myc-tagged p62 constructs were expressed in HEK cells followed by lysis in PD buffer (40 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.1% NP-40, 6 mM EDTA, 6 mM EGTA, 10 mM β -glycerophosphate, 10 mM NaF, 10 mM phenyl phosphate, 300 mM Na_3VO_4 , 2 mM PMSF, 10 μg of aprotinin/ml, 1 μg of leupeptin/ml, 1 μg of pepstatin/ml, 1 mM dithiothreitol). An equal amount of lysate (500 μg) was added to 10 μl of S5a agarose. Alternatively increasing concentrations of purified proteasome (0 to 1 μg) were added to GST-p62 beads (5 μg). The pull-down buffer was also supplemented with 0.5 mM ATP. The pull-downs were conducted in 500 μl of TBS-0.1% Tween 20 containing 5% glycerol. The pull-downs were performed for 2 h at 30°C followed by washing three times in TBS-0.1% Tween 20 with 5% glycerol. To the pelleted beads, 50 μl of SDS sample buffer was added followed by separation by SDS-PAGE and Western blotting analysis with antibody to myc to detect tagged p62 constructs captured by S5a. To detect proteasome interaction with GST-p62, blots were probed with antibody to Rpt1 (Affiniti). Alternatively, HEK cells were treated with interleukin-1 (IL-1; 10 ng/ml) and immunoprecipitated with p62 antibody followed by immunoblotting with Rpt1 antibody, or Rpt1 was immunoprecipitated and transfected p62 constructs were detected by blotting with myc antibody.

RESULTS

p62 binds to polyubiquitin through its C-terminal UBA domain and forms aggregates. By employing a bioinformatics approach, p62 has been shown to possess a UBA domain (16, 52), which is consistent with its ability to bind polyubiquitin chains (5, 53). However, the ability of p62 to bind and sequester polyubiquitinated substrates has not yet been firmly established. In addition, recent findings suggest that ubiquitin-interacting domains such as UBA and PUB may be necessary for ubiquitin sequestration into aggregates (11). For this study, three tagged constructs were employed (Fig. 1A) and are as follows: (i) the full-length p62; (ii) p62 Δ N-term, a construct missing amino acids 1 to 229; and (iii) p62 Δ UBA, a construct missing the UBA domain at amino acids 386 to 440. To establish the relationship between UBA polyubiquitin binding and aggregate formation, we examined whether p62 could bind polyubiquitin in vivo and if the UBA domain was required. Full-length p62 or a mutant lacking the UBA domain was coexpressed with HA-tagged ubiquitin in HEK cells (Fig. 1B). We observed that the UBA domain was necessary for p62 to interact with polyubiquitin. The coimmunoprecipitated ubiquitin (HA) signal was not due to the covalent attachment of ubiquitin chains to p62 itself, because the signal was lost when the cells were lysed in an SDS-containing lysis buffer. These findings corroborate other experiments (5, 53) demonstrating that the UBA domain of p62 is necessary for polyubiquitin binding, consistent with the function of other UBA domains (59). We noticed that cells expressing p62 possessed increased amounts of high-molecular-weight polyubiquitin. Thus, we tested if polyubiquitin accumulated in cells when p62 was overexpressed and a requirement for the UBA domain. The UBA domain of p62 was required for polyubiquitin sequestration, whereas its N terminus was not (data not shown). In parallel, p62 was able to sequester polyubiquitin in vivo. HEK cells were cotransfected with full-length p62, p62 lacking its UBA domain (p62 Δ UBA), or a construct lacking its N terminus (p62 Δ N-term) along with HA-tagged ubiquitin. The cotransfected cells were examined by confocal microscopy. Small aggregates of p62 colocalized with ubiquitin (Fig. 1C), which was dependant upon the presence of a UBA domain but not the N-terminal portion of p62. Aggregation may impair the proteasome or, alternatively, impaired proteasome activity may seed aggregates (2). If p62 plays a role in sequestering polyubiquitin,

TABLE 1. Relationship of p62 aggregates to cell survival^a

Treatment	% of cells			
	With aggregates		Without aggregates	
	Dead	Alive	Dead	Alive
Without ALLN	39	61	65	35
With ALLN	52	48	62	38

^a Cells were scored as aggregate positive or negative based upon the expression of GFP-p62. Cells were scored as dead or alive employing either the green or red stain (Molecular Probes).

impairment of the proteasome may enhance the formation of p62 aggregates containing polyubiquitin. Upon the inhibition of proteasomal degradation by treatment with ALLN (or MG132 [data not shown]), large aggregates of p62 which contained sequestered polyubiquitin were visible (Fig. 1C). The average number of aggregates per cell was essentially similar with or without ALLN; however, the size of the aggregates increased from 6 to 8 microns to 12 to 14 microns upon treatment with ALLN. Additionally, the formation of the p62 aggregates was dependent upon microtubules since treatment with either vinblastine or nocodazole prevented their formation (data not shown). In summary, through its UBA domain, p62 interacts with polyubiquitinated substrates, and the UBA domain is necessary for aggregate formation.

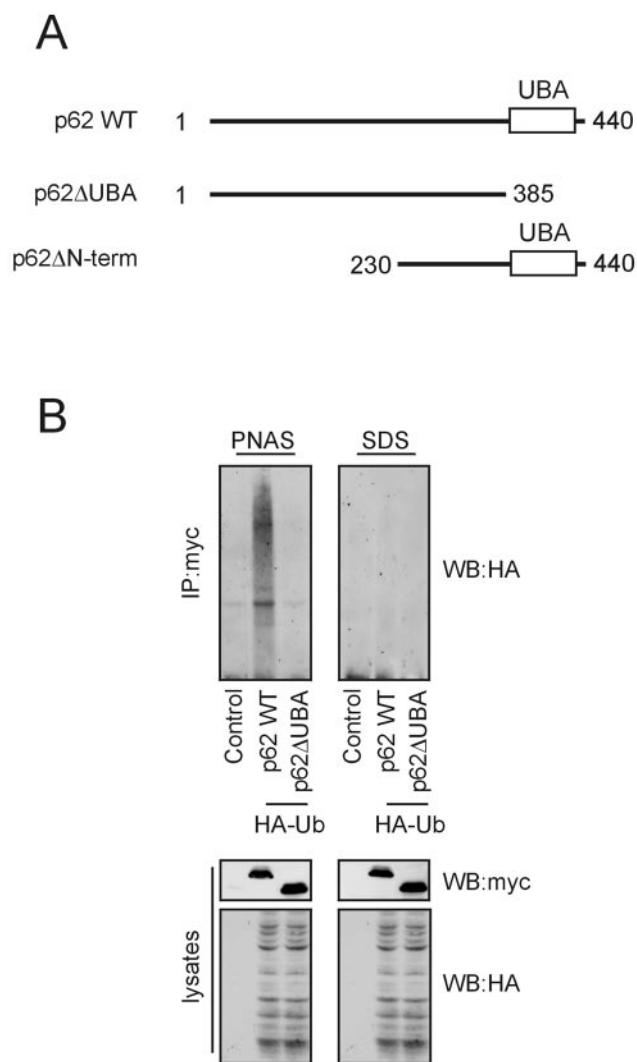
Aggregates compromise cell survival. The role of aggregates and their relationship to cell survival are controversial (1, 6). It has been suggested that aggregates represent the cell's attempt to rid itself of misfolded proteins, and hence, cells possessing aggregates represent a population of cells that are surviving, whereas cells without aggregates represent dying cells, although these observations may be condition and/or cell type specific. Since cells transfected with p62 lacking a UBA domain failed to form aggregates, this system provided us with a means to address the role of aggregate formation in relation to cell survival. Employing the Live/Dead Viability/Cytotoxicity assay, we first determined whether cells with p62 aggregates were dead or alive (Table 1). In cells lacking aggregates, there was essentially no difference in the survival profile in the presence or absence of ALLN treatment. Aggregate-containing cells consist of living cells, compared to those without aggregates. However, ALLN treatment decreased the number of living cells. We conclude that aggregates are a characteristic of living cells; however, proteasomal inhibition drives aggregate-containing cells away from survival. On the population level, similar results were observed when we used the MTS assay (Fig. 1D). Deletion of the UBA domain blocked the survival-promoting effects of p62. By comparison, deletion of the N-terminal region of p62 had less drastic effects on cell survival. Induction of p62 aggregates by treatment with ALLN completely inhibited the cell survival-promoting effects of p62 (Fig. 1D).

p62 interacts with K63-polyubiquitinated substrates through its UBA domain. Polyubiquitin chains may be formed in vivo by three linkages within ubiquitin: K29, K48, and K63 (45). Since each type chain may assume a different conformation (58), it has been suggested that each UBA domain may possess chain-specific recognition capabilities. To test the type of polyubiquitin chain recognized by the UBA domain of p62, HA-tagged wild-type ubiquitin or mutants of ubiquitin (K29R, K48R, and

K63R) were expressed in HEK cells and lysates interacted with GST-p62 UBA in a pull-down assay. As shown in Fig. 2A, the UBA domain of p62 interacted with HA-tagged polyubiquitin chains. However, when K63 of ubiquitin was mutated to arginine (R), the ability of polyubiquitin to bind the UBA domain of p62 was completely inhibited, whereas binding was retained when either K29 or K48 was mutated to R. Although present, K48R chain interaction was somewhat diminished upon mutation; however, this observation did not hold up in subsequent characterization studies (Fig. 2A, right top panel). In parallel, we further tested the interaction of the UBA domain of p62 with lysates prepared from cells expressing wild-type ubiquitin, K63R (lacking any K63-polyubiquitin chains), K29,48R (where the predominant pool of polyubiquitin expressed would be K63), or the triple mutant K29,48,63R (absence of the major polyubiquitin chains). Binding to the UBA domain of p62 was observed only with lysates from cells expressing HA-tagged wild-type ubiquitin or K29,48R (Fig. 2A). As a control, the addition of increasing concentrations of K48 chains into the binding assay failed to compete for binding with K29,48R polyubiquitinated substrates (data not shown). The UBA domain of p62 also directly bound in vitro-synthesized K63-polyubiquitin chains (S. Rassi and C. M. Pickart, personal communication). To assess the specificity of the p62 UBA pull-down assay at discriminating chain-specific interactions, we also tested the ability of hPLIC-2, a polyubiquitin-binding protein (30) whose UBA domain chain-binding properties have not yet been characterized, to bind polyubiquitinated substrates. Lysates prepared from cells expressing HA-tagged ubiquitin constructs revealed that the UBA domain of hPLIC-2 bound proteins with K48 chains but not those of K63 (data not shown). Collectively, these findings indicate that the UBA domain of p62 binds proteins with K63-linked polyubiquitin chains.

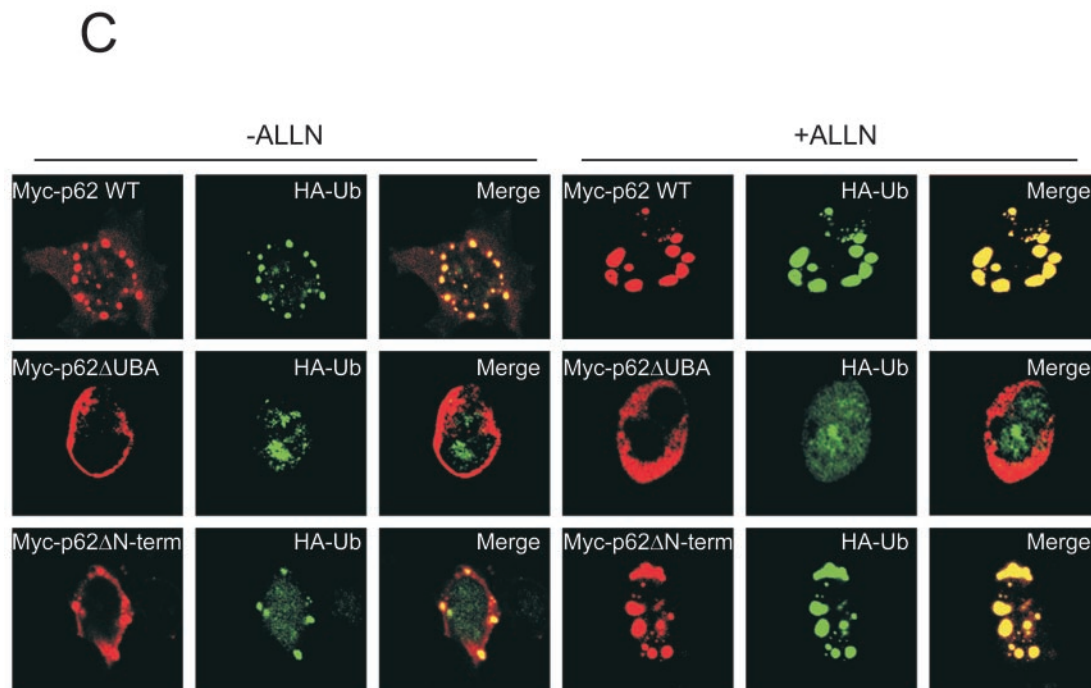
Since p62 possesses a binding site for TRAF6 (51), a RING finger E3 ubiquitin ligase (23), we suspected that TRAF6 may become trapped along with p62 and ubiquitin within the large aggregates upon inhibition of the proteasome. To test this idea, the colocalization of TRAF6 with p62 was examined in the absence or presence of proteasomal inhibition (Fig. 2B). The smaller punctate p62 aggregates possessed colocalized TRAF6, revealing that these structures likely serve as a microenvironment for TRAF6-p62 signaling (51). The larger insoluble aggregates formed upon inhibition of the proteasome likewise accumulated large amounts of TRAF6 (Fig. 2B), which were shown to colocalize with polyubiquitin (Fig. 1C).

As an E3 ligase, TRAF6 has been shown to selectively synthesize K63-polyubiquitin chains onto target substrates (8). p62 possesses a TRAF6-binding motif (60), and recent studies have shown that peptides homologous to this motif may competitively inhibit TRAF6 signaling or function (61). A peptide containing the TRAF6-binding motif present in p62 (underlined type) along with the hydrophobic sequence containing the cell-permeable motif (italicized type) from Kaposi fibroblast growth factor signal sequence was synthesized (*AAVA LLPAVLLALLAP-ESASGPS~~ED~~PSVNKLF*) or a control peptide with mutant amino acids in the interaction motif (*AAVA LLPAVLLALLAP-ESASGAS~~AD~~ASVNKLF*). Our studies reveal that the cell-permeable TRAF6 inhibitory peptide blocked p62-TRAF6 interaction in a dose-dependent fashion (Fig. 2C). We took advantage of the fact that IL-1 has been



shown to induce interaction of p62 with TRAF6 and activate TRAF6 signaling (51). HEK cells were treated or not with increasing doses of control or TRAF6 inhibitory peptide, followed by stimulation with IL-1. Lysates were prepared and included in a pull-down employing the UBA domain of p62. We hypothesized that should the p62-UBA domain bind K63-polyubiquitinated substrate proteins, a dose-dependent reduction in interaction of the lysates would occur with lysates prepared from cells pretreated with TRAF6 inhibitory peptide. In fact, this is what we found (Fig. 2C).

Since TRAF6 localized to the large aggregates along with polyubiquitin (Fig. 2B), we reasoned these aggregates may represent the failed attempt of the cell to rid itself of TRAF6-K63-polyubiquitinated substrates sequestered through interaction with the UBA domain. Hence, we reasoned that inhibition of TRAF6 interaction with p62 might prevent the formation of the aggregates. Pretreatment of cells with an inhibitory dose of TRAF6 peptide (but not control peptide [data not shown]) prior to treatment with ALLN resulted in a decrease in the size of the p62 aggregates (Fig. 2D). ALLN treatment caused 100% of the GFP-p62-expressing cells to accumulate large p62 aggregates. By comparison, treatment with TRAF6 peptide re-



D

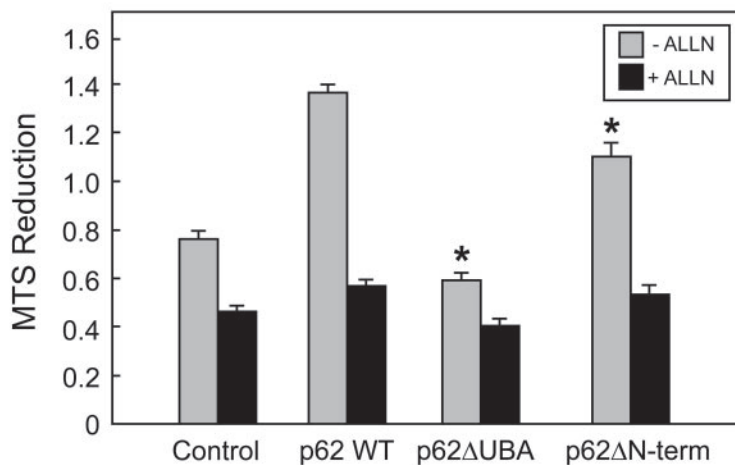
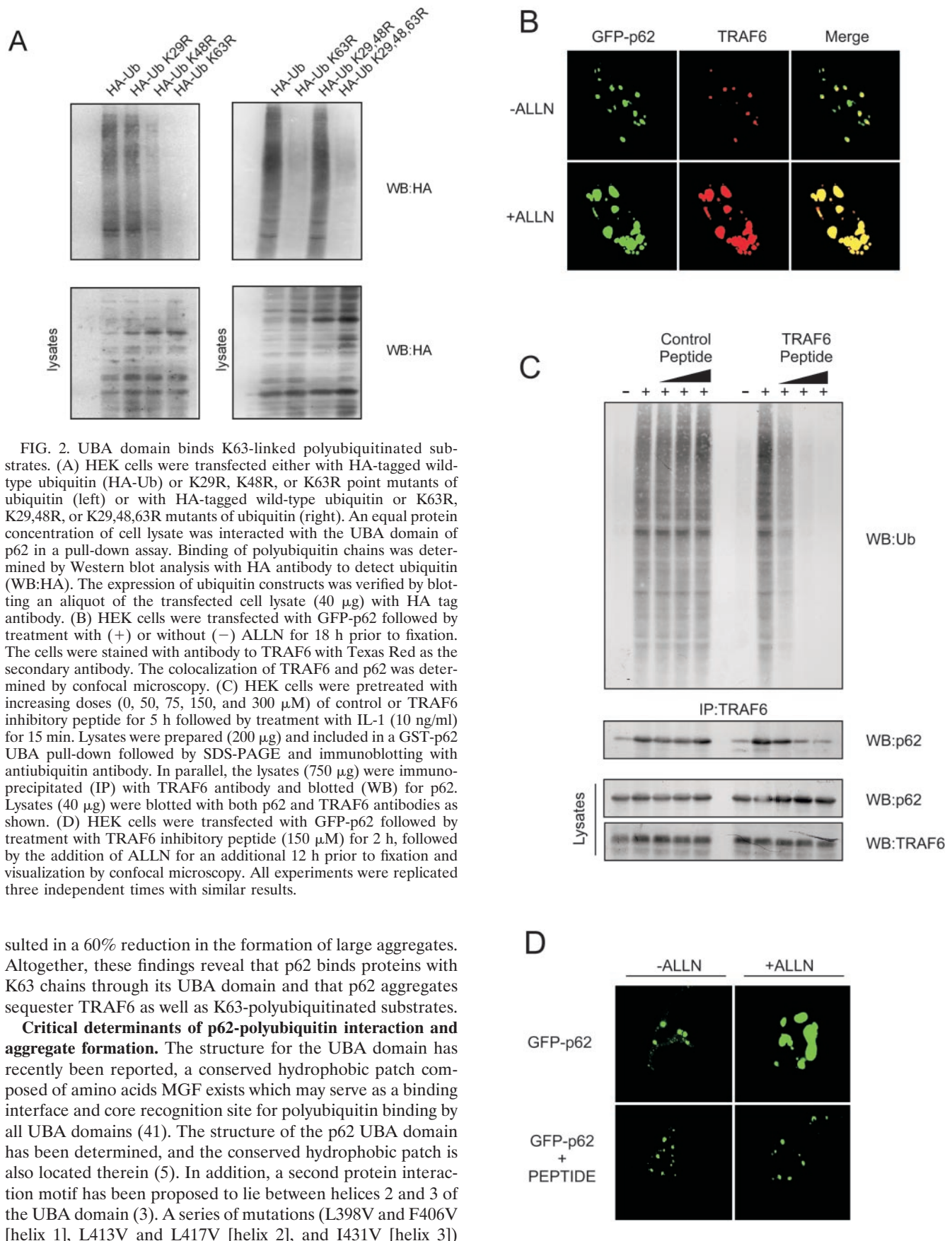


FIG. 1. UBA domain binds polyubiquitin noncovalently and is required for aggregate formation. (A) The p62 constructs employed were myc-tagged wild-type (WT) p62; full-length p62 (amino acids 1 to 440); p62 Δ N-term, a construct missing amino acids 1 to 229; and p62 Δ UBA, a construct missing the UBA domain (amino acids 386 to 440). (B) myc-tagged full-length p62 construct or myc-tagged construct lacking the UBA domain (p62 Δ UBA) were transfected into HEK cells along with HA-tagged ubiquitin (HA-Ub). Lysis and immunoprecipitation (IP) were carried out in either PNAS buffer (lacking SDS) or SDS lysis buffer (SDS) followed by SDS-PAGE and Western blotting (WB) for HA-tagged ubiquitin. (C) Shown are laser scanning confocal microscopy images of immunofluorescence staining for exogenous myc-p62 (red) and HA-ubiquitin (green) of wild-type myc-p62-expressing (top), myc-p62 Δ UBA-expressing (middle), and myc-p62 Δ N-term-expressing (bottom) HEK cells with (+) or without (–) proteasomal inhibitor ALLN (50 μ M) for 24 h. Cells were incubated with rabbit anti-myc IgG or mouse anti-HA IgG and labeled with Texas Red-conjugated anti-rabbit antibodies (red) or Oregon Green-conjugated anti-mouse antibodies (green), respectively. Merged images with overlapping immunoreactivity are shown in yellow. Note that cells expressing p62 lacking its UBA domain (p62 Δ UBA) fail to form aggregates or to colocalize with ubiquitin. All experiments were replicated three independent times with similar results. (D) HEK cells (in a 24-well plate) were transfected with myc-tagged wild-type p62, p62 Δ UBA, or p62 Δ N-term along with HA-tagged ubiquitin constructs. After 24 h of transfection, the cells were treated with or without ALLN (50 μ M) for 30 h. Cell survival was assessed by the addition of MTS reagent for 2 h. Values shown are means \pm standard error of the means of four different experiments. Survival was significantly diminished between the control and p62 Δ UBA ($P < 0.001$) and between wild-type p62 and p62 Δ N-term ($P < 0.001$).



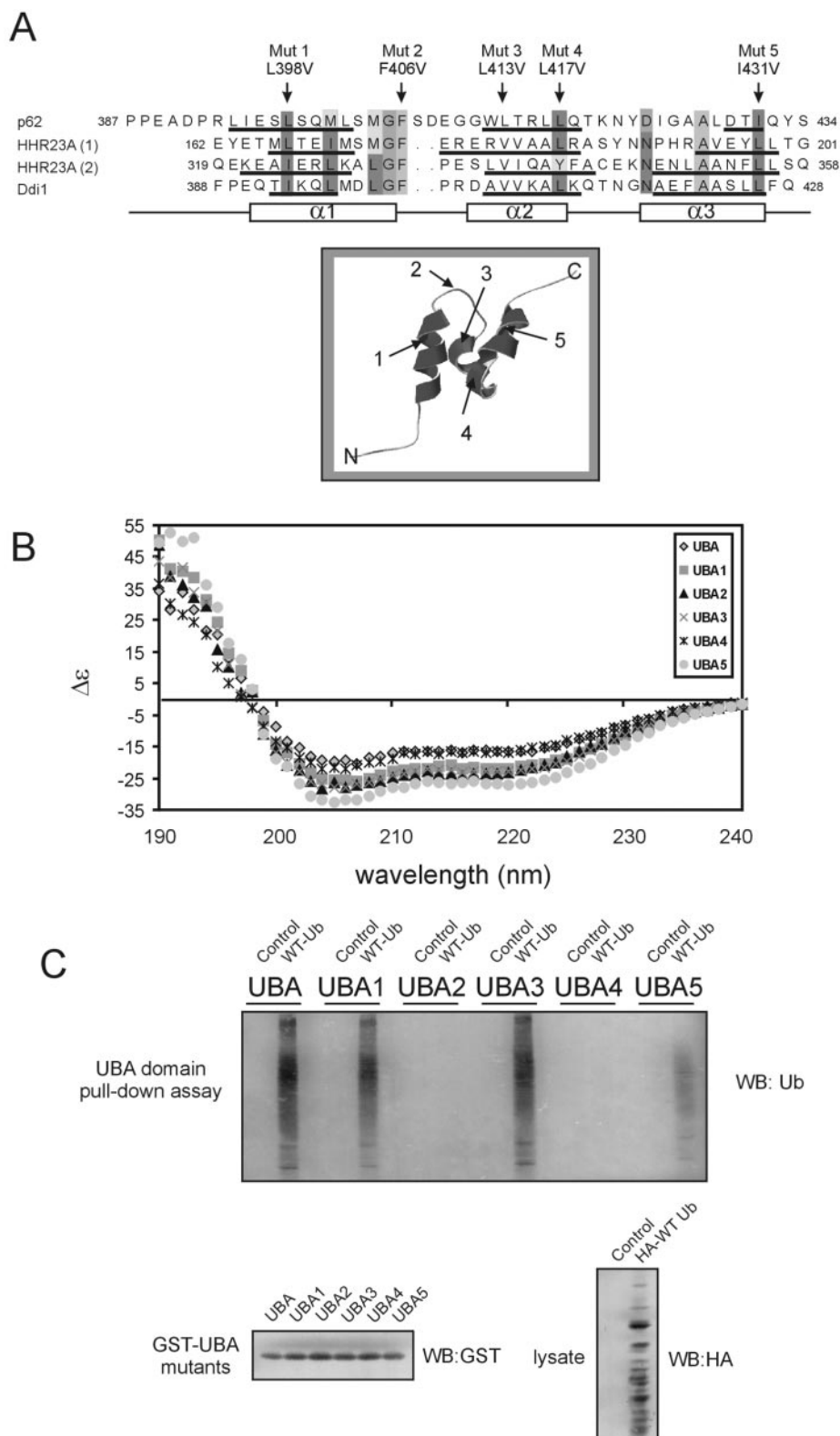


FIG. 3. Critical determinants of p62-polyubiquitin interaction. (A) Sequence alignment of the UBA domain of p62 with UBA containing proteins hHR23A UBA1, hHR23A UBA2, and Ddi1, indicating conserved residues and α -helix formation. Arrows indicate residues mutated for analysis as well as their location on a three-dimensional model of the p62 UBA domain. (B) Overlaid circular dichroism spectra of UBA peptides. (C) HEK cells were transfected with HA-tagged wild-type ubiquitin (WT-Ub) or left untransfected as the control. Pull-down assays were carried out by using the GST-UBA domain of p62 and its mutants UBA1, UBA2, UBA3, UBA4, and UBA5. The lysates (500 μ g) were interacted with 5 μ g of GST-UBA in a pull-down assay. The interaction of polyubiquitin chains with each UBA construct was analyzed by immunoblotting (WB) with antibody to ubiquitin. The findings were replicated three independent times with similar results. To validate equal amounts of GST-UBA in the pull-down assay, the blot (\sim 32kDa) was probed with GST antibody. The expression of HA-tagged ubiquitin was verified by immunoblotting with HA antibody.

TABLE 2. Estimation of secondary structures in UBA peptides based on CD data

Protein	Method of data analysis and average ^b	% of peptides with the following secondary structures ^a :					
		H _r	H _d	B _r	B _d	Turn	Coil
UBA (wild type)	CDSSTR	52.2	15.3	3.6	7.6	4.4	16.7
	CONTIN/LL	51.0	18.6	0.0	2.1	5.3	23.1
	Average	51.6	17.0	1.8	4.9	4.9	19.9
UBA1	CDSSTR	54.8	14.7	2.7	8.0	3.4	16.7
	CONTIN/LL	56.6	17.2	0.0	2.5	4.0	19.8
	Average	55.7	16.0	1.4	5.3	3.7	18.3
UBA2	CDSSTR	48.4	12.5	6.1	10.5	3.3	19.1
	CONTIN/LL	49.6	11.9	0.0	3.0	7.8	27.8
	Average	49.0	12.2	3.1	6.8	5.6	23.5
UBA3	CDSSTR	49.8	13.8	6.2	8.9	5.7	16.4
	CONTIN/LL	51.3	16.7	0.0	3.4	6.0	22.8
	Average	50.6	15.3	3.1	6.2	5.9	19.6
UBA4	CDSSTR	53.6	14.0	4.6	8.6	3.5	15.4
	CONTIN/LL	43.8	11.9	0.0	3.1	6.9	33.4
	Average	48.7	13.0	2.3	5.9	5.2	24.4
UBA5	CDSSTR	50.5	15.0	3.9	6.9	4.2	19.5
	CONTIN/LL	56.7	16.7	0.0	3.7	4.0	19.0
	Average	53.6	15.9	2.0	5.3	4.1	19.3

^a Abbreviations: H_r, regular α -helix; H_d, distorted α -helix (number of amino acids equal to or less than 4); B_r, regular β -strand; B_d, distorted β -strand (number of residues equal to or less than 2).

^b The readings of CONTIN/LL are the averages of the results from the CONTIN and CONTINLL programs. Average, average of the results from CDSSTR and CONTIN/LL methods.

within the p62 UBA domain were made (Fig. 3A) to assess residues that may be important for the structural integrity of the UBA domain and to determine which residues are necessary for polyubiquitin binding. In parallel, studies were undertaken to determine the effects that mutations in the UBA domain had upon the localization of p62 within aggregates and the ability of p62 to sequester polyubiquitin *in vivo*.

As a means to explore the consequences which each mutation had on perturbation of the secondary structure within the UBA domain, we first analyzed wild-type p62 and the five UBA domain mutants by employing CD spectroscopy. Since the UBA domain consists of three α -helical structures, the peptide from this domain was well suited for CD analysis. The CD spectrum of wild-type UBA has a band at 207 nm with $\Delta\epsilon_{\max}$ equal to $-20.38 \text{ M}^{-1} \text{ cm}^{-1}$ and a band at 220 nm with $\Delta\epsilon_{\max}$ equal to $-16.59 \text{ M}^{-1} \text{ cm}^{-1}$. This pattern is typical for proteins rich in helical content (4). The CD spectra of the mutants showed only small deviations from the wild-type UBA domain (Fig. 3B), indicating that the secondary structure of the mutants was not significantly perturbed. Consistent with this observation, secondary structural analyses of the experimental CD data using CDSSTR and CONTIN/LL programs (25, 46) resulted in essentially similar secondary structural contents for all the peptides (Table 2). Fits between the experimental CD data and the calculated data are good, with a root mean squared deviation in the range of 0.9 to 1.6.

The wild-type GST-p62 UBA domain along with the five UBA domain mutants were then employed in pull-down assays to assess their ability to interact with polyubiquitinated proteins. HA-tagged polyubiquitin was expressed in HEK cells,

and lysates were prepared and used as the source of polyubiquitinated substrates for a pull-down assay (Fig. 3C). The amount of the GST-tagged UBA domain mutants was equal in each pull-down, and the HA-tagged ubiquitin construct was effectively expressed. Mutant 2 (F406V) and mutant 4 (L417V) failed to bind polyubiquitin, with significantly reduced binding capacity exhibited by mutant 5 (I431V). Mutant 2 lies in the loop between helix 1 and helix 2 and is part of the MGF binding site (41) while mutant 4 lies in helix 2, indicating that polyubiquitin binding is not conferred by a single site, but both hydrophobic surfaces within the UBA domain appear to serve as effective recognition surfaces for polyubiquitin binding. In the case of mutant 4, even though both leucine and valine have helix-forming potential, valine is weaker than leucine. Interestingly, the CD analysis shows that there is also a slight decrease (about 7%) in the total helical content for both mutants compared to that of the wild type (Table 2). Thus, the loss of interaction between polyubiquitin and the UBA domain might be due to a loss of some secondary structure. These conclusions are somewhat tentative at this stage, and a detailed nuclear magnetic resonance analysis of the structures of these mutants will be required to rigorously establish these speculations.

Since the UBA domain is necessary for polyubiquitin binding and aggregate formation, we reasoned that the functional properties of the mutants could be assessed by examining their ability to sequester polyubiquitin along with the appearance of p62 within aggregates (Fig. 4). This approach would also provide another independent means to assess polyubiquitin chain interaction in an *in vivo* setting. The myc-tagged p62 constructs, the wild type and the five UBA domain mutants, were expressed in HEK cells along with HA-tagged ubiquitin and treated or not with the proteasomal inhibitor ALLN, followed by preparation for confocal microscopy. Effects of the UBA mutations on aggregate formation, p62 targeting to aggregates, and polyubiquitin sequestration fell into several classes. Compared to wild-type p62, mutants 1 and 3 had no effect on aggregate formation and on sequestration of polyubiquitin (Fig. 4). This is consistent with the ability of mutants 1 and 3 to effectively interact with polyubiquitin (Fig. 3C). By comparison, mutation of the core MGF hydrophobic interaction patch (mutant 2) completely inhibited these effects (Fig. 4), as did mutation of the second hydrophobic patch located in helix 2 (mutant 4). Interestingly, the inability of both mutant 2 and 4 to bind polyubiquitin *in vitro* (Fig. 3C) was confirmed *in vivo* by the lack of polyubiquitin sequestration with these two mutants. Mutant 5 was able to sequester polyubiquitin to a degree but failed to form large aggregates upon inhibition of the proteasome with ALLN (Fig. 4). The lack of large aggregate formation with mutant 5 is likely due to its diminished interaction with polyubiquitin chains (Fig. 3C). Collectively, these findings (Fig. 4) are consistent with the results obtained with the *in vitro* pull-down assay (Fig. 3C) and further support the hypothesis that large aggregates in proteasome-impaired cells result as a consequence of sequestration of K63-polyubiquitin substrates by the UBA domain of p62.

p62 is involved in ubiquitin proteasome-mediated degradation. Since aggregates have been reported to contain proteasomal components (35), it is possible that p62 may interact with the proteasome and be involved in the shuttling of substrates for degradation. To test this idea and to map the do-

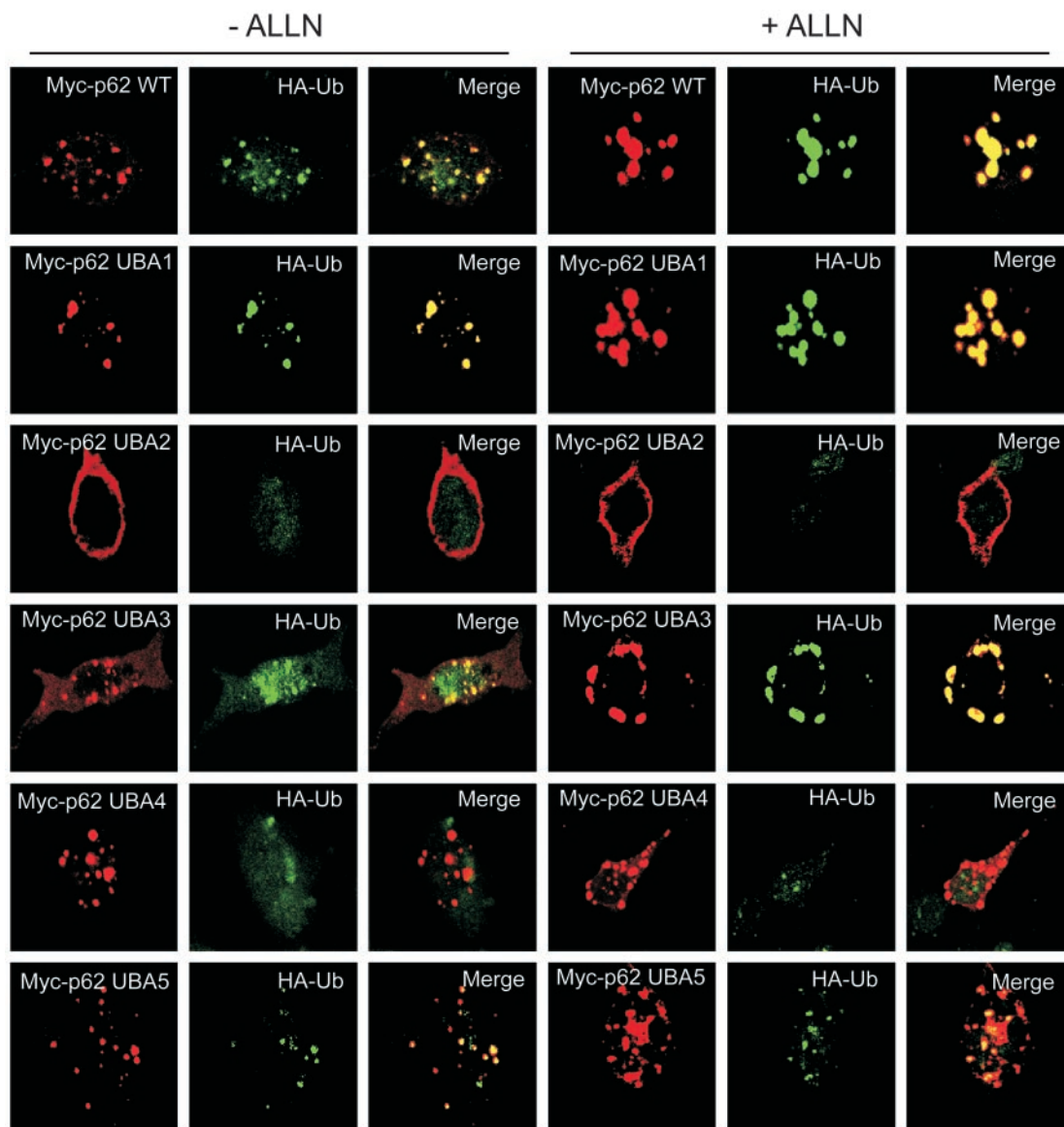


FIG. 4. UBA-polyubiquitin interaction is required for aggregate formation. HEK cells were cotransfected with HA-tagged ubiquitin (HA-Ub) together with myc-tagged full-length and UBA mutants of p62 expression constructs treated with (+) or without (–) proteasomal inhibitor ALLN (50 μ M) for 24 h and then labeled with antibodies to HA and *myc*. Images of a representative cell are shown. Anti-*myc* labeling (red) detects wild-type (WT) myc-p62 and its UBA domain mutants. Anti-HA labeling (green) detects ubiquitin. Merged images show the overlap between the individual staining patterns as yellow. The findings are representative of two independent experiments.

main that might be involved in mediating this interaction, full-length p62, p62 Δ N-term, and p62 Δ UBA were transfected into HEK cells and lysates were prepared and used for interaction with GST-S5a (Fig. 5A). Full-length p62 or the p62 construct missing the UBA domain cointeracted with S5a, whereas the N-terminal construct lacking the PB1 domain failed to interact with S5a. We conclude that the PB1 domain within amino acids 1 to 229 enables p62 to interact with S5a, a subunit component of the hinge of the 19S proteasomal particle (9, 14). This interaction may be indirect; therefore, additional experiments were undertaken to examine the ability of p62 to interact with the proteasome. We observed that p62 coprecipitated Rpt1 in a stimulus-dependent fashion (Fig. 5B, left). Alternatively, myc-tagged p62 constructs were transfected into HEK cells and the

interaction of Rpt1 and p62 was mapped. We observed that full-length p62 or p62 Δ UBA interacted with Rpt1, whereas p62 Δ N-term did not (Fig. 5B, right). Thus, the p62 N terminus directs interaction with the proteasome. Next, we tested whether p62 would bind 26S proteasomes directly in a pull-down assay. Indeed, GST-p62 was able to directly interact with the proteasome as indicated by the pull-down of Rpt1 (Fig. 5C). Alternatively, GFP-p62-transfected cells treated with ALLN were costained with antibody to either Rpn10/S5a or Rpt1 or with antibody to the “core” subunits of the 20S particle (Fig. 5D) to establish colocalization of p62 with the proteasome. Staining with any of the three antibodies resulted in colocalization with p62, whereas staining with LAMP-1, a lysosomal marker, failed to colocalize with p62 (data not shown).

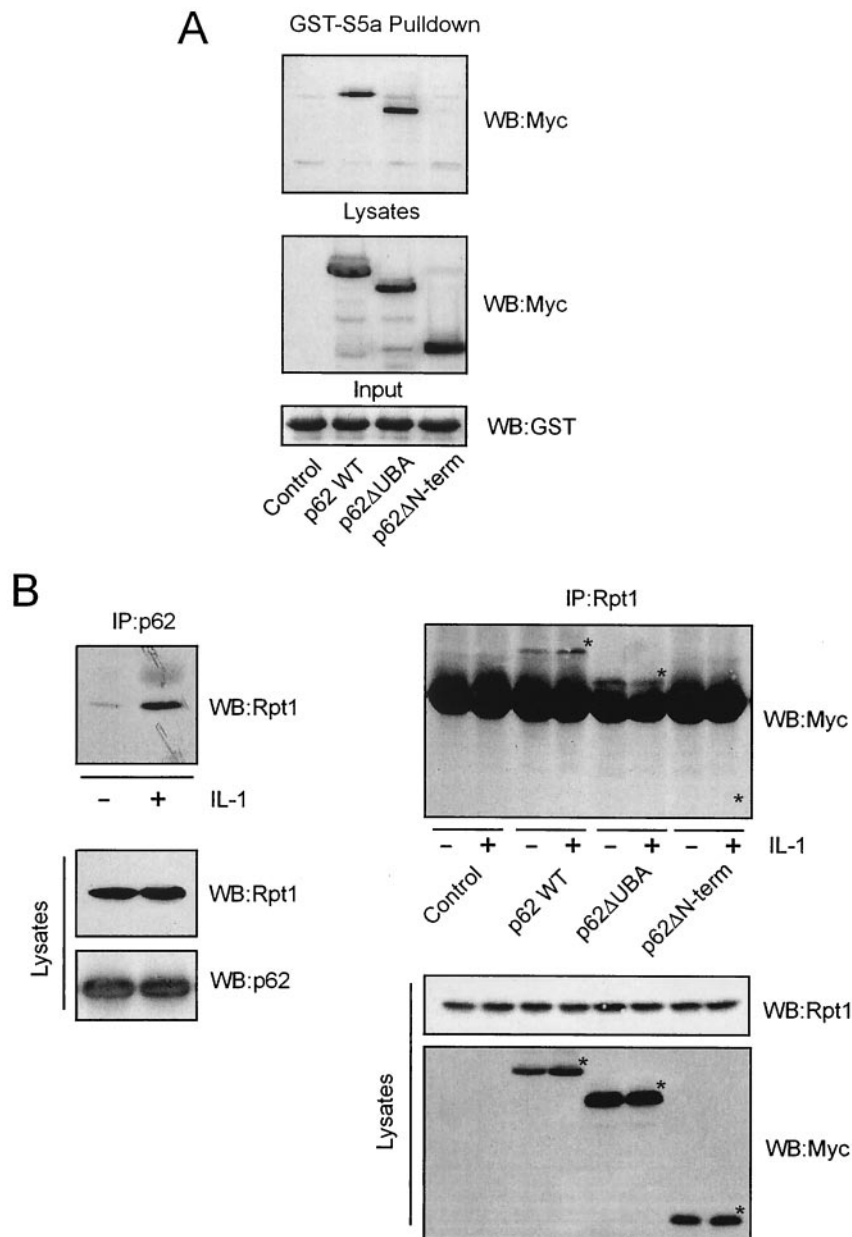


FIG. 5. p62 interacts with the proteasome. (A) myc-tagged constructs wild-type (WT) p62, p62ΔUBA, and p62ΔN-term were expressed in HEK cells, and lysates were prepared as described in Materials and Methods. An equal amount of GST-S5a agarose was added to 500 μ g of lysate from each sample and incubated for 2 h at 30°C. Following interaction, beads were washed, and myc-tagged constructs bound to S5a were analyzed by SDS-PAGE and Western blotting (WB) with myc antibody. Construct expression and the amount of GST-S5a input into the assay were also analyzed by Western blot with the appropriate antibody as shown. All experiments were replicated three independent times with similar results. (B) p62 interacts with proteasomes in a stimulus-dependent manner. HEK cells were treated with IL-1 (10 ng/ml) for 5 min followed by immunoprecipitation (IP) with p62 antibody and immunoblotting with Rpt1 (left). Alternatively, myc-tagged p62 constructs were expressed in HEK cells followed by stimulation with IL-1 (10 ng/ml). The lysates (750 μ g) were immunoprecipitated with Rpt1 antibody and immunoblotted with myc to detect the tagged p62 constructs (right; shown with an asterisk). As the control, the expression of the proteins in the lysate (40 μ g) was determined. (C) p62 interacts directly with the proteasome. Immobilized bacterially expressed GST-p62 (5 μ g) or GST control was incubated with purified 26S proteasomes (0 to 1 μ g). After washing, bound subunit Rpt1 was detected by immunoblotting. (D) p62 aggregates contain proteasomal subunits. HEK cells were cotransfected with GFP-p62 and treated with ALLN (50 μ M) for 18 h and then labeled with antibodies to Rpn10/S5a, Rpt1, or proteasomal subunits as indicated. Images of a representative cell are shown. Green detects the GFP-p62 and red detects the endogenous proteins. Merged images show the overlap between the individual staining patterns in yellow.

Since p62 captures polyubiquitinated substrates through its UBA domain and interacts with the proteasome through its N terminus, we hypothesized that p62 may be involved in the shuttling of polyubiquitinated substrates for degradation by the

proteasome. We reasoned that depletion of p62 levels might therefore result in inhibition of ubiquitin proteasome-mediated degradation and an accumulation of ubiquitinated proteins. To test this idea, we transfected HEK cells with a full-

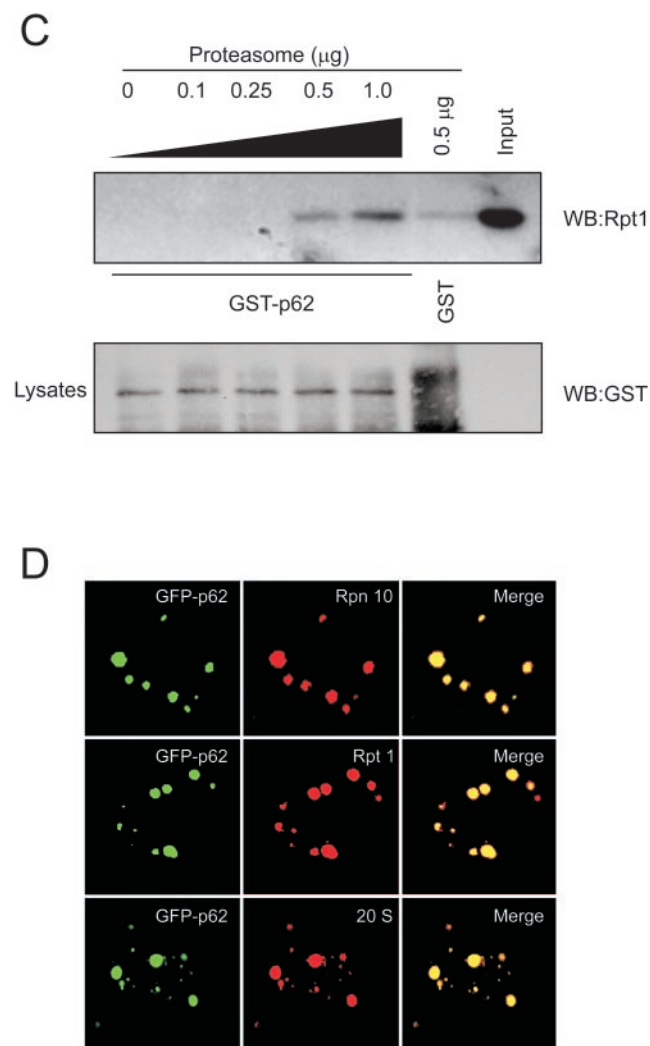


FIG. 5—Continued.

length antisense p62 construct (60) which was followed by treatment of cells with cycloheximide to prevent protein synthesis and examine protein turnover. Transfection of the antisense p62 construct reduced endogenous p62 levels by 80% (Fig. 6A). Control cells rapidly turned over several substrates (Fig. 2B and C). By comparison of five prominent polyubiquitinated substrates, depletion of p62 levels resulted in the accumulation of these proteins. These findings reveal that p62 serves a newly described role in ubiquitin-mediated proteolysis and may serve as a shuttling factor in the delivery of polyubiquitinated substrates for proteasomal degradation.

DISCUSSION

In this study, we demonstrate that the p62 UBA domain interacts specifically with substrates possessing K63-polyubiquitin chains, while other UBA domains of Rad23, Dsk2, Ddi1, hHR23A, and hPLIC-2 (12, 15, 48) interact with K48 chains, and UBA interaction with K29 chains has been reported (24). Both K29 (24) and K48 (15, 48) chains have been reported to serve as proteolytic degradation signals. In comparison, K63-polyubiquitin chains are generally regarded as playing a role

outside of degradation, such as in the activation of the NF- κ B pathway (8) and in DNA damage tolerance (54). The ability of p62 to interact with the proteasome reveals that under certain circumstances, p62 may serve as a shuttling factor to deliver K63-polyubiquitinated substrates for proteolytic degradation. In this regard, depletion of p62 retards the turnover or degradation of several polyubiquitinated proteins. Previous studies employing in vitro-synthesized K48 and K63 chains (22) observed that K63 chains could serve as a competent signal for degradation by proteasomes. That some substrates which are K63 polyubiquitinated (8, 54) are not degraded suggests that other coassociating factors may determine whether the K63-polyubiquitin signal serves as a degradation signal. The role of p62 as a proteasomal shuttling factor will unfold as specific K63-polyubiquitinated substrates that interact with the p62 UBA domain are discovered.

It is not yet apparent which amino acids impart chain-specific recognition for a given UBA domain. Since each type of chain may adopt a particular conformation (57, 58), the critical amino acids that likely define chain-specific recognition are those that contribute to the overall compactness of the UBA domain and the accessibility of the hydrophobic interaction surfaces with a particular type chain of a given conformation. In addition, the length of the polyubiquitin chain itself likely plays a role in recognition by the polyubiquitin receptor (43). In this regard, another study recently observed that the p62 UBA domain interacted with in vitro-synthesized K48 chains (5). It must be noted that our assays have been conducted with chains linked to substrates, which is different from an analysis of p62 interaction with chains alone. Our study further reveals that in addition to the hydrophobic patch site between α -helix 1 and α -helix 2, there clearly exists a second interaction domain that lies in helix 2. Studies with other UBA domains have suggested this to be the case as well (3). Clearly, much remains to be understood about the nature of the UBA domain, its ability to discriminate chain-specific conformations, and its ability to bind polyubiquitinated proteins.

We propose that p62 functions in a fashion analogous to that of other proposed shuttling factors which bind polyubiquitinated substrates through their C-terminal UBA domain and, via an N-terminal ubiquitin-like (UBL) domain, interact with the proteasome (12, 38, 48). Interestingly, the solution structure of the UBL domain has been solved and is similar to that of the β -sheet conformation of ubiquitin (42). In this regard, the PB1 motif of the p62 N terminus is structurally similar, assuming a compact $\beta\beta\alpha$ -ubiquitin fold (62). Thus, PB1 and UBL motifs may share redundant functional properties. The structural similarity of the p62 PB1 domain (34) to the UBL domain (42), which is known to interact with S5a (20), provides a plausible explanation for the ability of p62 to interact with S5a. The interaction of p62 with the proteasome would also explain the ability of proteasome inhibitors to increase the size of p62 aggregates, since p62 itself did not possess covalently linked ubiquitin. Interestingly, the p62 N-terminal PB1 domain also interacts with both aPKC (40) and MEK5 (10, 34). Thus, one could envision that the release in one of the components of a ternary complex formed between aPKC, MEK5, and p62 (34) might regulate p62 interaction with the proteasome.

There has been interest in understanding the molecular ba-

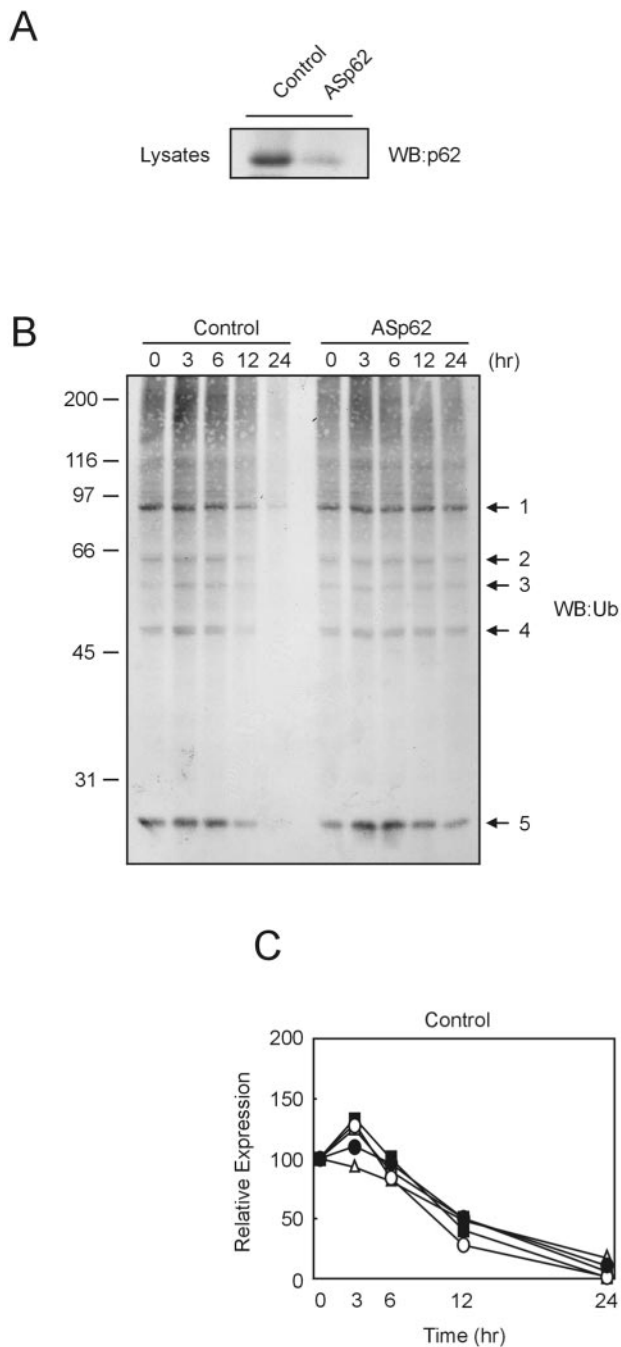


FIG. 6. p62 is involved in ubiquitin proteasome degradation of polyubiquitinated proteins. (A) HEK cells were transfected with full-length antisense p62 (ASp62) construct or not. The levels of endogenous p62 were examined by immunoblotting lysates (40 μ g) with p62 antibody. (B) Control or antisense p62-transfected HEK cells were treated with 20 mg of cycloheximide/ml as indicated. Whole-cell lysates (40 μ g) were separated by SDS-PAGE and immunoblotted (WB) with antiubiquitin antibody. (C) The relative expression of five prominent polyubiquitinated proteins present in both control and antisense p62 lysates was determined and plotted as shown. The experiment was repeated twice with similar results.

sis of aggregation, particularly with respect to neurodegeneration (1, 6). In this regard, proteins with a polyubiquitin binding (PUB) motif are required for the localization of ataxin-3 to poly(Q) aggregates (11). Recruitment of the unrelated ubiquitin-binding protein sequestosome 1/p62 into aggregates was also mediated by its UBA domain (11). Our findings herein extend these observations and reveal not only that aggregates recruit p62 but also that recruitment to the aggregate is dependent upon the ability of the UBA domain to sequester polyubiquitin. Aggregates are reported as being dynamic structures (29). Small aggregates could serve as the microenvironment for the recruitment of multimeric signaling complexes.

Previous studies have shown that TRAF6, IRAK, and p62 colocalize into small aggregates, dependant upon IL-1 stimulation (51). Similar aggregates have been observed in nerve growth factor-stimulated cells as well (49). Overexpression of a protein may impair the function of the proteasome and serve to seed aggregates (2). Alternatively, in certain disease states, the activity of the proteasome may be impaired or diminished by an age-related mechanism or by specific effects upon the proteasome itself. Such appears to be the case with Alzheimer's disease, where amyloid β ($A\beta$) (18) or mutant ubiquitin (UBB^{+1}) has been shown to block proteasome activity (33). Whatever the exact cause or mechanism, brains of individuals

with Alzheimer's disease possess decreased proteasome activity (28). In either case, prosurvival signaling complexes could seed aggregates containing trapped polyubiquitinated substrates. Interestingly, p62 has been colocalized with ubiquitin, tau, and A β peptide in tangle structures obtained from Alzheimer's brain (31, 32), but is sparse or absent from healthy tissue. We speculate that through impaired proteasomal function, TRAF6-K63-polyubiquitinated substrates might be recruited into p62 aggregates and may contribute to the neurodegenerative disease process. In this manner, proteins that provide normal prosurvival functions would be depleted from the cell.

The discovery of the proteins that interact with the p62 UBA domain will provide further insight into the functional aspects of this protein in the context of various signaling cascades. K63-polyubiquitinated substrates play a critical role in development, since the absence of TRAF6 is lethal (37). Recent studies have revealed that mutations in the UBA domain of p62 segregate with Paget's disease, a disorder characterized by risk of fracture, neurological complications, and increased risk of osteosarcoma (21, 26, 36). Several mutations in the p62 UBA domain, including point mutation P392L, a premature stop codon resulting in a protein lacking a UBA domain or mutation in the splice site, have been observed in persons with this disease. Since the UBA domain of p62 is necessary for interaction with K63-polyubiquitinated substrates, our findings suggest that the failure of specific polyubiquitinated substrates to interact with the p62 UBA domain may contribute to the pathophysiology of Paget's disease.

In conclusion, our findings reveal that the N terminus of p62 targets its interaction with the proteasome, and the C-terminal UBA domain (amino acids 386 to 440) interacts with K63-polyubiquitinated substrates. Altogether, our results reveal a novel role for this protein as a shuttling factor for delivery of polyubiquitinated substrates to the proteasome for degradation. Different splice variants of p62 exist, one lacking a TRAF6 binding site (17) and one lacking the C-terminal UBA domain (7); therefore, several types of complexes may exist in vivo and function in a context- as well as a tissue-specific manner. Future studies in p62 knockout mice (13) should clarify some of the important issues relative to the function of this protein.

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