RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis

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Summary

We have used RNA interference (RNAi) to examine the functional relationship between valosin-containing protein (VCP/p97/Cdc48p/TER94) ATPase and the ubiquitin-proteasome system (UPS) in *Drosophila* S2 and human HeLa cells. In both cell types, RNAi of VCP (and, to a lesser extent, of certain VCP-interacting proteins) caused significant accumulation of high-molecular-weight conjugates of ubiquitin, an indication of inhibited UPS function. However, decreased VCP levels did not directly inhibit proteasome activity. In HeLa cells, polyubiquitinated proteins accumulated as dispersed aggregates rather than as single aggresomes, even in the presence of proteasome inhibitors, which normally promote aggresome formation. RNAi of VCP caused extensive vacuolization of the cytoplasm, and proteasome inhibitors exaggerated this feature. RNAi of VCP had little effect on S2 cell proliferation but blocked cell-cycle progression and induced mitotic abnormalities and apoptosis in HeLa cells. These results indicate that VCP plays an important general role in mediating the function of the UPS, probably by interacting with potential proteasome substrates before they are degraded by the proteasome.

Supplemental data available online

Key words: VCP, Ubiquitin, Proteasome, Proteolysis, Aggresome

Introduction

The ubiquitin-proteasome system (UPS) is responsible for the constitutive degradation of most cellular proteins and regulates a remarkably large and diverse group of cellular processes by conditionally degrading proteins that control or are essential for those processes (Glickman and Ciechanover, 2002). Substrates of the UPS are covalently modified with a polyubiquitin chain, which targets them for degradation by the 26S proteasome (Pickart, 2001). This large protease complex is composed of two functionally distinct subcomplexes: the 20S proteasome and the PA700 regulatory complex (19S cap, RP) (Coux et al., 1996; DeMartino and Slaughter, 1999; Zwickl et al., 2001). The cylinder-shaped 20S proteasome catalyzes peptide bond hydrolysis at multiple catalytic sites located near the center of an axial channel through its interior. PA700 binds to one or both ends of the 20S proteasome cylinder and mediates proteolysis by: (i) binding the polyubiquitin chain (Lam et al., 2002; Deveraux et al., 1994); (ii) unfolding the protein substrate (Braun et al., 1999; Strickland et al., 2000; Liu et al., 2002); (iii) opening 'gates' at the entrances of the axial channels (Kohler et al., 2001); (iv) translocating the unfolded polypeptide chain through the open gates to the internal catalytic sites (Lee et al., 2001); and (v) removing the polyubiquitin chain from the substrate (Yao and Cohen, 2002). Degradation of ubiquitinated proteins by the 26S proteasome requires continuous ATP hydrolysis. Several of the PA700-mediated subfunctions listed above are regulated by ATP and have been linked to one or more of six distinct PA700 subunits which are ATPases of the AAA family (ATPases associated with multiple cellular activities) (Ogura and Wilkinson, 2001).

Among the many crucial cellular roles of the UPS is protein quality control, whereby proteins with abnormal structures because of genetic mutations, thermal or oxidative damage, or errors in synthesis are selectively degraded (Garcia-Mata et al., 2002). Some abnormal proteins, however, are refractory to salvation by chaperones or destruction by the UPS and form highly insoluble aggregates. Aggregate formation is especially pronounced under certain pathological conditions referred to as 'conformational diseases' (Kopito, 2000; Kopito and Ron, 2000) and can be induced experimentally by protein overexpression and/or by pharmacological inhibition of the proteasome. Under these conditions, aggregated proteins coalesce into a single large structure (Wojcik et al., 1996b; Wojcik, 1997) recently termed the 'aggresome' (Johnston et al., 1998). Aggresomes form by dynein-driven, microtubule-dependent centripetal transport of peripheral aggregates to an area surrounding the centrosome (Wojcik et al., 1996b; Johnston et al., 1998; Garcia-Mata et al., 1999). Aggresomes also contain chaperones, such as Hsp70, and many components of the UPS, including proteasomes and polyubiquitin conjugated to the protein aggregates (Wojcik et al., 1996b; Wigley et al., 1999; Fabunmi et al., 2000).

Proteins that are residents of or that transit through the endoplasmic reticulum (ER) are subject to a mechanistically specialized category of quality control termed ER-associated degradation (ERAD) (Hampton, 2002). Misfolded ER proteins are expelled from the ER, probably through the Sec61 channel, and degraded in the cytoplasm by the UPS. Although many molecular details of ERAD remain unknown, recent work has identified valosin-containing protein (VCP) as a crucial cytoplasmic component of the process (Ye et al., 2001; Bays et al., 2001; Braun et al., 2002; Rabinovich et al., 2002; Jarosch et al., 2002). VCP is a widely distributed AAA ATPase also known as p97 in mammals and amphibians (Peters et al., 1990), TER94 in insects (Pinter et al., 1998), cdc48 in yeast (Moir et al., 1982; Frohlich et al., 1991) and VAT in Archaea (Pamnani et al., 1997). VCP is a ring-shaped homohexamer of a 97 kDa protein that contains two copies of the conserved ATP-binding domain characteristic of AAA family members and an N-terminal polyubiquitin-binding domain (Peters et al., 1990; Dai and Li, 2001). Because ERAD substrates are ubiquitinated before they are fully released into the cytoplasm, VCP might couple its ATPase activity to extraction of ubiquitinated substrates as they emerge from the Sec61 channel (Bays and Hampton, 2002). VCP also has been implicated in two types of ERAD involving normal proteins, including a novel example of limited proteolysis responsible for activation of the SPT23 transcription factor and the constitutive turnover of several short-lived ER membrane proteins (Rape et al., 2001). In the former instance, proteasome-catalysed limited endoproteolysis of one subunit of ER membrane bound, homodimeric SPT23 produces a soluble ubiquitinated fragment (p90), which then functions as a nuclear transcription factor. Remarkably, VCP is required for dissociation of the cleaved ubiquitinated p90 from its uncleaved partner. These results indicate that VCP can function as an ubiquitin-specific 'segregase' (Rape et al., 2001; Braun et al., 2002).

In addition to its role in ERAD, VCP has been identified as a mediator of numerous other cellular functions including membrane fusion (Latterich et al., 1995; Meyer et al., 1998; Kondo et al., 1997; Rabouille et al., 1998), nuclear trafficking (Hetzer et al., 2001) and cell proliferation at the level of both cell division and apoptosis (Frohlich et al., 1991; Shirogane et al., 1999; Asai et al., 2002). In many instances, specific adaptor proteins with which VCP forms multiprotein complexes direct its role in a given process. For example, VCP's various roles in ERAD are mediated by the Ufd1-Npl4 heterodimer (Bays et al., 2001; Braun et al., 2002). *Ufd1* was originally identified as a gene responsible for the degradation of ubiquitin fusion proteins (Johnson et al., 1995). Recent work has shown that Npl4 binds polyubiquitin via a zinc-finger domain (Meyer et al., 2002). By contrast, VCP's role in Golgi membrane fusion is mediated by p47 homotrimers (Kondo et al., 1997; Meyer et al., 1998). p47 binds ubiquitinated proteins through its UBA domain (Meyer et al., 2002). Yeast VCP (cdc48) also interacts physically with several other proteins required for degradation of ubiquitin fusion substrates. One such protein, Ufd2, is a polyubiquitin chain elongation factor (E4) (Koegl et al., 1999), whereas another, Ufd3, is of unknown function (Ghislain et al., 1996). These results suggest a close relationship between VCP and UPS function. The precise role of VCP in the regulation of apoptosis is unclear but could be related to the role of the UPS in this process (Wojcik, 2002). For example, Ufd2 is cleaved by caspase during apoptosis (Mahoney et al., 2002).

Recently, we have used RNA interference (RNAi) to examine the functional roles of individual proteins of the UPS (Wojcik and DeMartino, 2002). This method is particularly useful for the study of essential genes, such as those of most components of the UPS, because those proteins can be eliminated from existing populations of cells. In this report, we have targeted VCP and some of its adaptor proteins for RNAi to understand the functional relationships of these proteins to both UPS function and the multiple cellular roles ascribed to VCP. Our results demonstrate several novel cellular phenotypes caused by elimination of VCP and suggest that VCP complexes are important mediators of UPS function.

Materials and Methods

Nomenclature and database searches

To simplify the nomenclature, we have denoted *Drosophila melanogaster* homologues of the proteins of interest by their mammalian names and the prefix 'd'. Alternative nomenclature of *Drosophila* proteins is provided in Table 1. Human proteins of interest were identified in the NCBI database and used to identify respective *D. melanogaster* homologues using PSI-BLAST (Altschul et al., 1997; Marchler-Bauer et al., 2002).

Chemical reagents and antibodies

Anti-VCP monoclonal antibody was from BD Transduction Laboratories (San Diego, CA). Anti-ubiquitin rabbit polyclonal serum and anti-α-tubulin monoclonal antibody were from Sigma (St Louis, MO). The FK2 monoclonal antibody against polyubiquitinated and monoubiquitinated proteins was from Biotrend (Cologne, Germany). Anti-pericentrin rabbit polyclonal serum was from BabCO/Covance (Berkeley, CA). Anti-PARP antibody was from Roche Applied Science (Indianapolis, IN). Anti-p53 mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-COP polyclonal serum was from Affinity BioReagents (Golden, CO). Anti-TGN46 sheep polyclonal serum was from Serotec (Raleigh, NC). Anti-hsp70 and anti-BIP antibodies were from Stressgen (Victoria, Canada). MCP72 anti-α2 monoclonal antibody was a generous gift from K. Hendil (August Kronegh Institutet, Copenhagen, Denmark) (Kaltoft et al., 1992). Anti-p47 polyclonal serum was a gift from G. Warren (Yale University, New Haven, CT) (Hetzer et al., 2001). Anti-p62 (CLIMP-63) polyclonal rabbit serum was a gift from D. Mundy (UT Southwestern, Dallas, TX) (Mundy and Warren, 1992; Mundy, 1995). Anti-dVCP/TER94 antiserum was a gift from D. McKearin (UT Southwestern, Dallas, TX) (Leon and McKearin, 1999). Horseradish peroxidase conjugated sheep anti-mouse whole IgG was from Amersham Biosciences (Piscataway, NJ). Anti-horseradish-peroxidase goat anti-rabbit whole IgG was from American Qualex (San Clemente, CA). FITC- and TRITC-conjugated anti-rabbit and anti-mouse Fab′ fragments were from Jackson Immunoresearch (West Grove, PA).

All the cell culture media and reagents were from Invitrogen Gibco (Carlsbad, CA). Yo-PRO iodide was from Molecular Probes (Eugene, OR). Lactacystin and MG132 were from Calbiochem (San Diego, CA). Suc-Leu-Leu-Val-Tyr-AMC was from Bachem (Bubendorf, Switzerland).

Double-stranded RNA synthesis and RNA interference in *Drosophila* S2 cells

Schneider 2 (S2) cells were grown as described previously (Wojcik

Table 1. Forward and reverse primers used to synthesize dsRNA for RNAi in *Drosophila* S-2 cells (T7 promoter sequence not shown)

Targeted protein	Accession number	Primers
dVCP (TER94)	AE003831	(F) 5′-TAAGACGCTGCTGGCCAA-3′ (R) 5′-CATCGCGGAGTTCTGATTTT-3′
dp47 (CG11139)	AE003841	(F) 5′-AGCAAGTGCATCGGCATC-3′ (R) 5′-CGGATCGTCATAGTGACGAA-3′
dUfd1 (CG6233)	AE003546	(F) 5′-TCCAATGCTGTTCAAGCTGA-3′ (R) 5′-ATTTCGCCGCCAACAGTT-3′
dUfd2 (CG9934)	AE003638	(F) 5′-TTCCTTTCCGTGTCGCTGTT-3′ (R) 5′-AGGCAGGTAGCCCAGATGAT-3′
dNpl4 (CG4673)	AE003752	(F) 5′-TGGAGCGCTTCCTCAACTACT-3′ (R) 5′-ATCAGACAATTATCGCGCAC-3′

and DeMartino, 2002). Double-stranded RNA (dsRNA) was synthesized as described previously (Wojcik and DeMartino, 2002). All primers were purchased from Qiagen Operon Technologies (Alameda, CA) (Table 1). The quality of dsRNA was assessed by electrophoresis and the final concentration of dsRNA was adjusted to 3 μg ml^{-1}. RNAi in S2 cells was performed as described previously (Wojcik and DeMartino, 2002).

Design and synthesis of small interfering RNAs

Small interfering RNAs (siRNAs) were designed using the algorithm proposed by Elbashir et al. (Elbashir et al., 2001) using mRNA sequences shown in Table 2. The specificities of sequences were confirmed by BLAST search against the NCBI databases (Altschul et al., 1997). The siRNAs were synthesized, purified and annealed by Dharmacon Research (Lafayette, CO) or synthesized using the Silencer™ siRNA construction kit (Ambion, Austin, TX) according to manufacturer's instructions. Those siRNAs synthesized by chemical means were dissolved in RNase free water at 20 mM concentration, whereas the siRNAs synthesized by the use of the Silencer kit were dissolved at 2 mM concentration and stored at –20°C.

Cell culture and transfection of HeLa cells

HeLa cells were grown in Dulbecco's modified Eagle's medium, a high-glucose medium, supplemented with 10% foetal calf serum and antibiotic/antimycotic solution. For RNAi experiments, cells were seeded either in six-well, 24-well or 96-well cell culture plates, or in two-chamber Labtek II slides (Nunc Nalgene, Naperville, IL) 24 hours before the transfection. For each well on a six-well plate or a chamber on the Labtek slides, 5 μl of the stock siRNA solution was mixed with 1.5 μM Oligofectamine™ reagent (Invitrogen, Carlbad, CA) and 93.5 μl Opti-MEM medium. After 20 minutes, the cells were washed and transfected in a total volume of 500 μl of Opti-MEM. After 4 hours, the cells were supplemented with 1.5 ml normal culture media per well. For wells on 24-well or 96-well plates, the amounts of all reagents were reduced 1:2 or 1:10, respectively. After 2 days of culture, siRNA transfection was usually repeated using the same procedure.

SDS-PAGE and western blotting

PBS-washed HeLa cells were lysed for 30 minutes in RIPA buffer supplemented with Complete™ Mini protease inhibitor mix (Roche Molecular Biochemicals, Indianapolis, IN). After measuring the protein concentration (Bradford, 1976), samples were diluted with 5× SDS-PAGE sample buffer (Laemmli, 1970), subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose and blotted with the relevant antibodies using standard methods. Detection was achieved using ECL™ Plus western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Images were scanned using the Storm Imager from Molecular Dynamics. Densitometry was performed using the ImageQuant software v. 5.2 from Molecular Dynamics (Amersham Bioisciences, Piscataway, NJ).

Cell growth

Cell growth was assessed by the MTT assay as described previously (Mosmann, 1983; Wojcik and DeMartino, 2002). In brief, this colorimetric assay measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in intact cells. Because reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of cell viability.

Determination of hydrolytic activity of the proteasome

Proteasome activity was assessed by measuring rates of hydrolysis of Suc-Leu-Leu-Val-Tyr-7-amido-methylcoumarin (AMC). HeLa cells lysates were prepared in 50 mM Tris-HCl, pH 7.6, and cleared by centrifugation, and the supernatants were used to determine the protein concentration (Bradford, 1976) and enzymatic activity, as described previously (Wojcik and DeMartino, 2002).

Determination of caspase 3/7 activity

Caspase 3/7 activity was determined using the Apo-ONE™ homogenous assay (Promega, Madison, WI) according to manufacturer's instructions.

Flow cytometry

Flow-cytometric analysis of the cell cycle was performed as described previously (Wojcik and DeMartino, 2002). Fluorescence was measured using a FACScan flow cytometer, data were analysed using CellQuest software (Beckton Dickinson, Palo Alto, CA).

Immunofluorescence confocal microscopy

HeLa cells were grown in Labtek two-chamber slides (Nunc Nalgene, Naperville, IL), fixed with methanol at –20°C for 10 minutes, washed twice for 15 minutes each with TBS, pH 7.6, supplemented with 0.1% bovine serum albumin and 0.1% fish gelatin, and incubated with primary antibodies diluted in the same buffer containing Tween-20 for 2 hours. After three 15 minute washes in TBS with 0.1% bovine serum albumin and 0.1% fish gelatin, the cells were incubated with secondary fluorescein- or rhodamine-conjugated anti-rabbit and/or anti-mouse Fab′ fragments. When needed, DNA was counterstained

Table 2. Sequences of the sense strands of the siRNAs used for RNAi interference in HeLa cells

Targeted protein	Accession number	Sequence	Method of synthesis
β5	D29011	5′-AAGAAGGUGAUAGAGAUCAAC-3′	Chemical
VCP	NM_007126	5′-AAGUAGGGUAUGAUGACAUUG-3′	Silencer
Ufd1	AF141201	5′-AAUGACAGGUCAGAUGUGGAG-3′	Silencer
Npl4	NP_060391	5′-AAGUCUUUGGCGCUCCCAACG-3′	Silencer
p47	NM_016143	5′-AAGAUGUUCAUGUAGUAUUGA-3′	Silencer

with 1 μM Yo-PRO™ iodide in TBS after a 30 minute RNAse digestion. After two washes in TBS, cells were mounted using Gel/Mount (Biomeda, Foster City, CA). Optical sections were acquired using the Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany).

Online supplemental material

The online supplemental material consists of four figures (Figs S1–S4, http://jcs.biologists.org/supplemental/) that contain the single-channel images of Fig. 5.

Results

RNAi of VCP and its adaptor proteins in *Drosophila* cells

To study the role of VCP in UPS function, we first used RNAi to reduce *Drosophila* VCP (dVCP) levels in *Drosophila* S2 cells. RNAi reduced *dVCP* mRNA by 75% and dVCP protein levels by 80% (Fig. 1). RNAi of dVCP promoted an almost twofold accumulation of ubiquitinated proteins, a general indicator of inhibited UPS function (Fig. 1B), but had no detectable effects on cell proliferation or general cellular morphology (as judged by light microscopy and by the MTT assay, data not shown). VCP forms at least two distinct complexes with alternative adaptor proteins: VCP^{p47} and $VCP^{Ufd1\text{-}Npl4}$ (Meyer et al., 2000). Moreover, VCP interacts with Ufd2, a protein originally identified as a component of the ubiquitin-fusion/protein-degradation pathway of the UPS (Meyer et al., 2000; Johnson et al., 1995). Therefore, we also used RNAi to determine the relative roles of these VCP-interacting proteins in UPS function. RNAi completely suppressed the production of *dp47* mRNA, substantially suppressed the production of *dUfd1* and *dUfd2* mRNAs, and suppressed the production of *dNpl4* mRNA only modestly (Fig. 1A). We were unable to confirm corresponding reductions in any of the respective proteins because suitable antibodies against these *Drosophila* proteins are not available. Nevertheless, RNAi of dUfd1 and dNpl4 significantly increased levels of ubiquitinated S2 cell proteins, albeit to a lesser extent than did RNAi of VCP (Fig. 1B). By contrast, RNAi of p47 and Ufd2 had little effect on the level of ubiquitinated S2 cell proteins. RNAi of these various VCP-interacting proteins had no detectable effect on cell proliferation or morphology. These data demonstrate the involvement of VCP and certain of its adaptor proteins in the UPS function in *Drosophila*, and prompted us to investigate this issue further in a mammalian cell system.

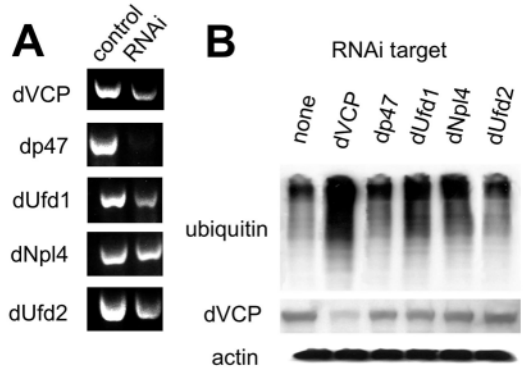

Fig. 1. RNAi of VCP and VCP adaptor proteins in *Drosophila* S2 cells. *Drosophila* S2 cells were subjected to RNAi of the indicated proteins. (A) The mRNA levels of indicated RNAi targets were determined by semiquantitative reverse-transcription PCR 4 days after the addition of indicated dsRNAs. (B) Equal amounts of whole-cell lysate protein were subjected to western blotting using antibodies against ubiquitin, dVCP and actin.

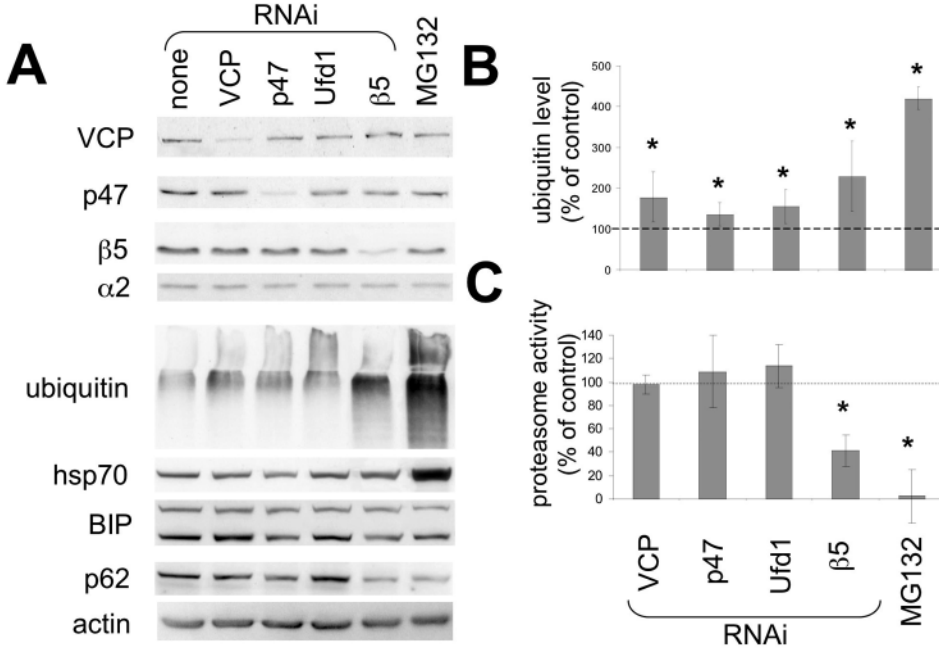

Fig. 2. RNAi of VCP, p47, Ufd1 and β5 subunit of the proteasome in HeLa cells. HeLa cells were subjected to RNAi of the indicated proteins for 4 days or treated with 5 μM MG132 for 24 hours. (A) Equal amounts of whole cell lysate protein were subjected to western blotting with the indicated antibodies. The anti-BIP antibody detects both BIP/Grp78 (lower band) and Grp94 (upper band); (B) Ubiquitin levels were quantified by densitometry of western blots. The ubiquitin level from control lysates was assigned a value of 100% and the levels from experimental lysates are expressed as a percentage of the control. Results represent mean values (±s.e.m.) from three independent experiments. The value for each experimental group was compared to the control by Student's *t* test. The asterisk indicates $P<0.05$. (C) Lysates were assayed for the chymotrypsin-like activity of the proteasome by hydrolysis of Suc-LLVY-AMC. Activities from control lysates were assigned a value of 100% and activities from experimental samples are expressed as a percentage of the control. Results represent mean values (±s.e.m.) of three independent experiments. Each experimental group was compared to the control by Student's *t* test. The asterisk indicates $P<0.05$.

RNAi of VCP and VCP adaptor proteins in human cells

To target VCP for RNAi in HeLa cells, we tested five different siRNAs. Each of these siRNAs decreased the level of VCP protein but they did so to different extents (data not shown). For all experiments described below, we used the most efficient siRNA (Table 2), which reduced VCP levels by 80±15%. RNAi of VCP caused an approximately twofold increase in the levels of ubiquitinated HeLa cell proteins (Fig. 2A,B). Thus, these effects were similar to those of RNAi in S2 cells. We also targeted human p47 and Ufd1 for RNAi. RNAi reduced p47 levels by 85±5% (Fig. 2A) and caused a small but significant increase (~25%) in ubiquitinated proteins (Fig. 2B). RNAi of Ufd1 also caused a small but significant increase in the level of ubiquitinated HeLa cell proteins (Fig. 2B) but lack of a suitable antibody against mammalian Ufd1 prevented us from determining the level of reduction of this protein. Proteasome activity was not affected by RNAi of any of these proteins (Fig. 2C), indicating that the effect on UPS function was not via direct inhibition of the protease.

To assess further the effects of RNAi of VCP and its adaptor proteins, we compared them to those caused by inhibition of UPS function by RNAi of the β5 subunit of the proteasome and by pharmacological inhibition of the proteasome. RNAi of β5 reduced β5 levels by 60±25%, inhibited proteasome activity by 60% and caused a threefold accumulation of ubiquitinated proteins. By comparison, treatment of HeLa cells with the proteasome inhibitor MG132 for 24 hours inhibited proteasome activity by 95% and caused a fivefold increase in the level of ubiquitinated proteins (Fig. 2). Whereas RNAi of p47 or Ufd1 had no effect on cell morphology, RNAi of VCP and β5 caused significant and similar changes, including decreased cell growth, elongation of some cells and rounding and detachment of others (see below).

In total, these results provide strong support for VCP-mediated degradation of a quantitatively significant subset of UPS substrates in mammalian cells, and suggest that each of two different VCP-containing complexes ($VCP^{Ufd1\text{-}Npl4}$ and VCP^{p47}) has a role in regulation of UPS function, although the magnitude and nature of these roles appear to differ.

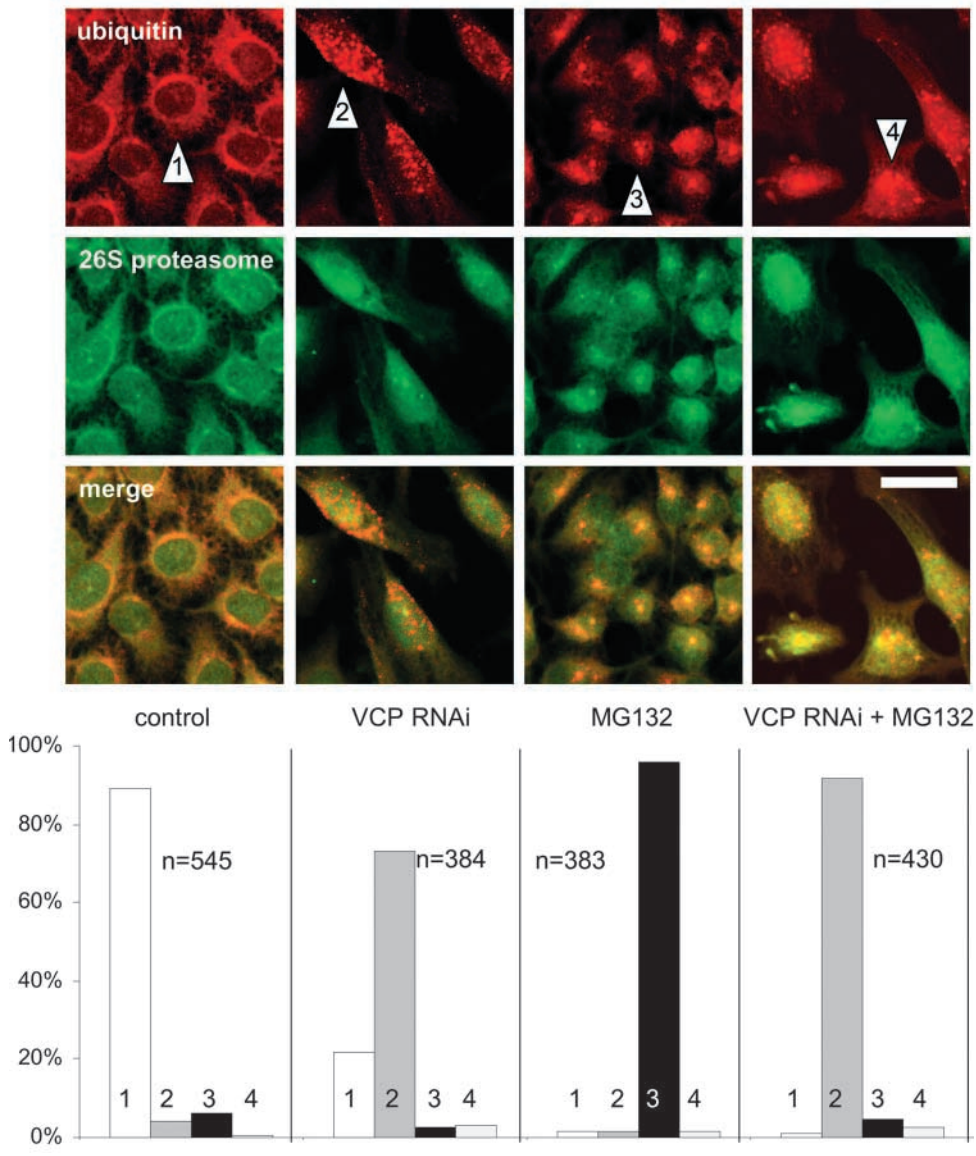

Fig. 3. RNAi of VCP alters the distribution of ubiquitinated proteins and inhibits formation of aggresomes. HeLa cells were subjected to RNAi of VCP for 4 days and/or treated with 10 μM MG132 for 6 hours.
(Top) Immunofluorescence confocal micrographs of the indicated experimental groups was conducted with antibodies against either mono- and polyubiquitinated proteins (FK2 antibody) (red) or the Rpn12 subunit of the 26S proteasome (green).
(Bottom) Cells for the control and each experimental group were scored for the number of cells with one of four characteristic ubiquitin labelling patterns: (1) no aggregates; (2) dispersed aggregates; (3) single aggresomes; and (4) heavy aggregates. A typical cell for each labelling pattern is indicated by the correspondingly identified arrowhead in the top row of the top panel. The relative abundance of each labelling pattern within a given group of cells is expressed as a percentage of scored cells (*n* indicates the number of scored cells). The distribution of patterns within each group is significantly different from another with $P<0.05$ based on a χ^2 test. Scale bar, 20 μm.

RNAi of VCP in HeLa cells induces dispersed aggregates of ubiquitinated proteins and prevents aggresome formation

Cells treated with proteasome inhibitors accumulate polyubiquitinated proteins and UPS components around the centrosome in a structure termed the aggresome (Wojcik, 1997; Wojcik et al., 1996b; Wigley et al., 1999; Johnston et al., 1998). To determine whether the polyubiquitinated proteins that accumulate as a consequence of RNAi of VCP also form an aggresome, we used immunofluorescence confocal microscopy with antibodies against polyubiquitin, a subunit of the 26S proteasome and pericentrin (a centrosomal marker). In over 90% of control cells, polyubiquitinated proteins and 26S proteasomes were diffusely distributed in the cytoplasm and nucleus (Fig. 3). Small aggresomes were observed in 5% of control cells (Fig. 3), as reported previously (Wigley et al., 1999). Also as reported previously, treatment of HeLa cells with MG132 promoted the formation of large single aggresomes in nearly all cells, as judged by the co-localization of polyubiquitin, 26S proteasome and pericentrin (Figs 3, 4). 75% of cells subjected to RNAi of VCP also demonstrated localized foci of polyubiquitinated proteins but, in contrast to aggregates formed by MG132 treatment, these foci were smaller, more numerous and highly dispersed throughout the cells (Figs 3, 4). Moreover, these dispersed foci did not localize with the 26S proteasome or markers for the centrosome. 20% of cells subjected to RNAi of VCP had dispersed ubiquitin labelling (Fig. 3). These cells probably represent the population that had not been transfected efficiently with *VCP* siRNA. Our results suggest that VCP might be required for the formation of authentic aggresomes. To test this possibility, HeLa cells subjected to RNAi of VCP were also treated with MG132. Over 90% of these cells also failed to form single discrete aggresomes and had a phenotype similar to that of those only subjected to VCP RNAi (Figs 3, 4).

RNAi of VCP in HeLa cells causes vacuolization of the cytoplasm

To understand the role of VCP in the dynamics of aggresome formation, we examined VCP subcellular localization by immunofluorescence confocal microscopy. In control HeLa cells, VCP was found in a perinuclear structure that co-localized with the Golgi markers β-COP and TGN46 but not with the ER membrane marker p62 or the centrosomal markers γ-tubulin and pericentrin (Figs 5, 6, and data not shown).

The localization of VCP was not altered appreciably in cells treated with MG132, with the VCP often seeming to surround or juxtapose the aggresome as previously reported for Golgi apparatus (Wojcik et al., 1996b). As expected, the intensity of VCP labelling was markedly reduced in cells subjected to RNAi of VCP. The residual VCP appeared more dispersed than in control cells and occasionally had a nuclear localization whose significance is unclear. The Golgi apparatus was also more dispersed in these cells, as judged by the labelling pattern of β-COP and TGN46 (Fig. 5). However, the most striking feature of the cells subjected to RNAi of VCP was vacuolization of the cytoplasm (Figs 5, 6). The p62 antibody labelled the periphery of these vacuoles, suggesting that these structures arise from distension of the ER cisternae. Although less prominent vacuolization was sometimes observed in cells treated only with MG132, cells treated with MG132 during RNAi of VCP showed significantly greater vacuolization than did cells with either treatment alone (Figs 5, 6).

To investigate further the nature of the vacuolar structures induced by RNAi of VCP, cells were co-labelled for p62 and the intralumenal ER chaperone BIP (Fig. 6). As expected, control cells showed appreciable co-localization of p62 and BIP, although some BIP-positive areas, especially around the nucleus, were devoid of p62 labelling. MG132 treatment did not alter this pattern. RNAi of VCP caused increased BIP labelling

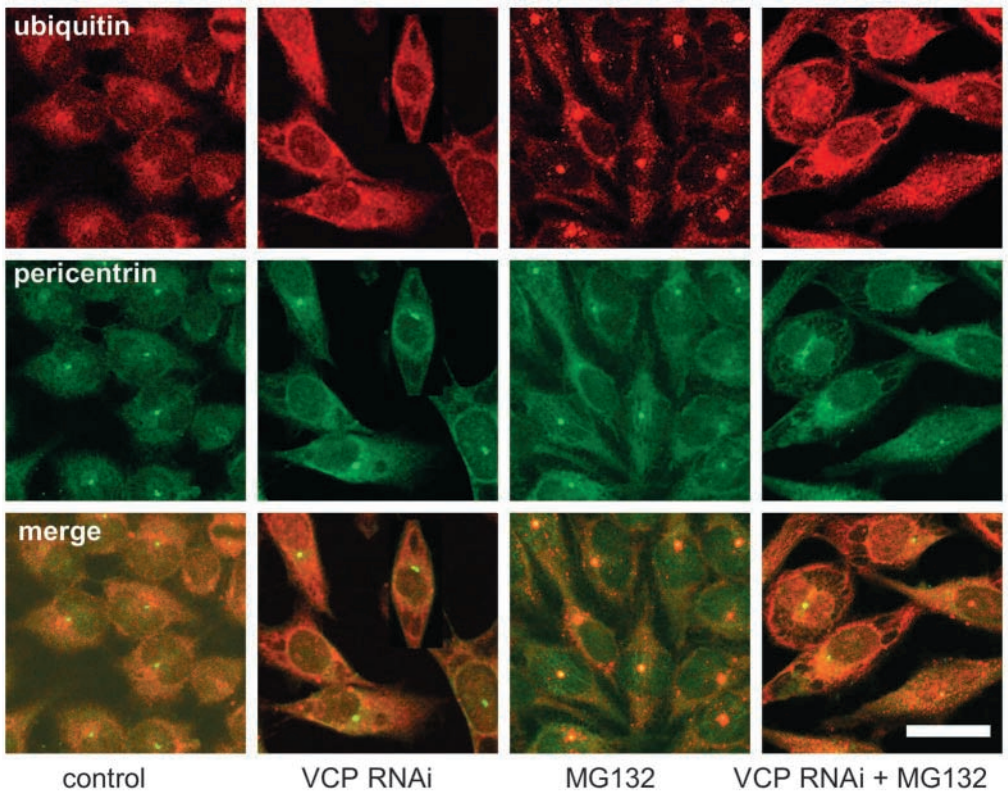

Fig. 4. RNAi of VCP prevents the accumulation of ubiquitinated proteins at the centrosome. HeLa cells were subjected to RNAi of VCP for 4 days and/or treated with 10 μM MG132 for 6 hours. Immunofluorescence confocal microscopy was conducted with the FK2 antibody detecting mono- and polyubiquitinated proteins (red) and anti-pericentrin antibody detecting the centrosome (green). Scale bar, 20 μM.

at the periphery of RNAi-induced vacuoles, as well as some labelling within their lumen. Moreover, p62 (which retained its ER membrane localization) became enriched around the nucleus and was often observed within the nucleus. Similar distribution patterns of these proteins were observed in cells subjected to RNAi of VCP that were also treated with MG132. These results demonstrate that reduction of VCP promotes significant alterations in the structure of the ER and Golgi apparatus, which leads to the vacuolization of large portions of the cytoplasm.

In contrast to RNAi of VCP, RNAi of p47, Ufd1 and Ufd2 did not produce appreciable changes in the distribution of ubiquitinated proteins in HeLa cells. Moreover, these treatments did not induce vacuole formation and had no effect on MG132-induced aggresome formation (data not shown). Although these results suggest that certain phenotypes caused by RNAi of VCP differ from those caused by RNAi of VCP adaptor proteins, meaningful comparisons about the relative functions of these various proteins cannot be made until the extent of all RNAi knockdowns is defined.

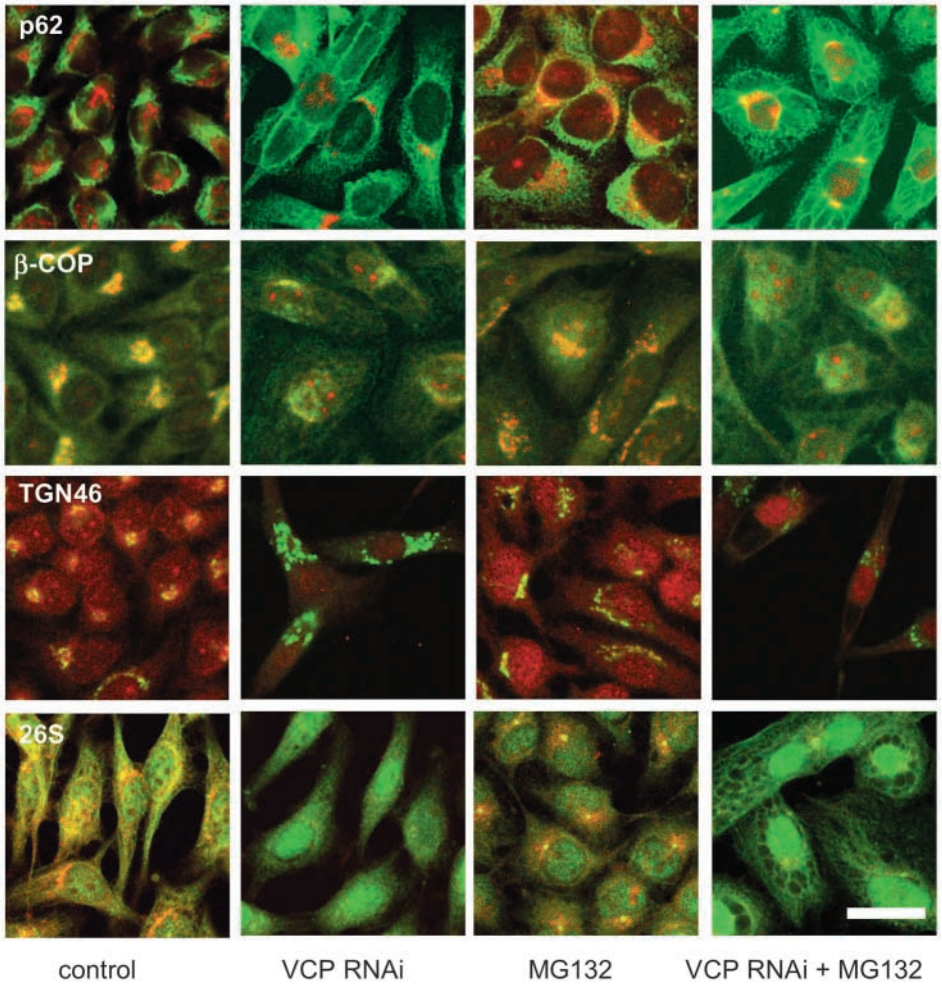

Fig. 5. RNAi of VCP induces vacuolization of the cytoplasm. HeLa cells were subjected to RNAi of VCP for 4 days and/or treated with 10 μM MG132 for 6 hours. Immunofluorescence confocal microscopy was conduced for VCP (red) and for p62 (an ER marker protein), β-COP (a cis-Golgi marker protein), TGN46 (a trans-Golgi marker protein) and Rpn12 (a subunit of the 26S proteasome) (green). Merged images are shown. Single channel images are available online (Figs S1, S2, S3 and S4, http://jcs.biologists.org/supplemental/). Scale bar, 20 μm.

RNAi of VCP in HeLa cells impairs cell proliferation

As described above, RNAi of VCP in HeLa cells reduced cell proliferation. To examine this effect further, we assessed cell cycle progression by flow cytometry. RNAi of VCP produced a block in the S and G2/M phases of the cell cycle with a concomitant decrease in the G0/G1 phase (Table 3). This effect is specific for reduction of VCP, because cells treated with only transfection reagent or cells that were transfected with several irrelevant siRNAs had normal cell cycle distributions (Table 3 and data not shown). At the level of decreased expression achieved here, RNAi of p47 or Ufd1 in HeLa cells had no significant effect on cell cycle progression (data not shown). By contrast, cells subjected to RNAi of the β5 subunit of the proteasome or cells treated with MG132 had alterations similar to, but less pronounced than, that of RNAi of VCP (Table 3). To extend the evaluation of the effects of reduced VCP on cell proliferation, we examined mitotic cells after 2 days of RNAi using the DNA dye Yo-PRO (Fig. 7). RNAi of VCP caused an accumulation of cells arrested in an aberrant stage of prometaphase/metaphase. More than 90% of the mitotic cells were abnormal, as opposed to fewer than 5% abnormal control cells treated with transfection medium only. The mitotic abnormalities consisted of the lack of mitotic progression beyond prometaphase/metaphase, a low degree of chromatin condensation and spindle defects including spindle fibres that were abnormally short, dispersed and/or compacted. Many cells were also characterized by large blebs, as visualized by immunofluorescence microscopy with an anti-α-tubulin antibody (Fig. 7, arrows). After 4 days of RNAi, the number of mitotic cells decreased significantly, and correlated with decreased levels of cyclin B1 (Fig. 8A).

RNAi of VCP in HeLa cells promotes apoptosis

Decreased proliferation, mitotic abnormalities, vacuolization, detachment from the substratum and bleb formation are all features of cells undergoing apoptosis. Moreover, previous work has established that inhibition of proteasome activity and/or accumulation of ubiquitinated proteins promote apoptosis (Wojcik, 2002). We therefore examined the effect of RNAi of VCP on activation of caspases 3/7 and cleavage of

PARP, two sensitive indicators of apoptosis. RNAi of VCP significantly increased caspase 3/7 activity (Fig. 8B) and promoted cleavage of PARP (Fig. 8A), indicating that VCP deficiency promotes apoptosis. Flow cytometry revealed cell debris with DNA content lower than 2C, an indication of the possible presence of apoptotic bodies (Table 3). To extend these findings, we examined the levels of the proapoptotic protein p53. RNAi of VCP caused a large and progressive increase in p53 levels during treatment, providing further support for induction of apoptosis during this treatment (Fig. 8A).

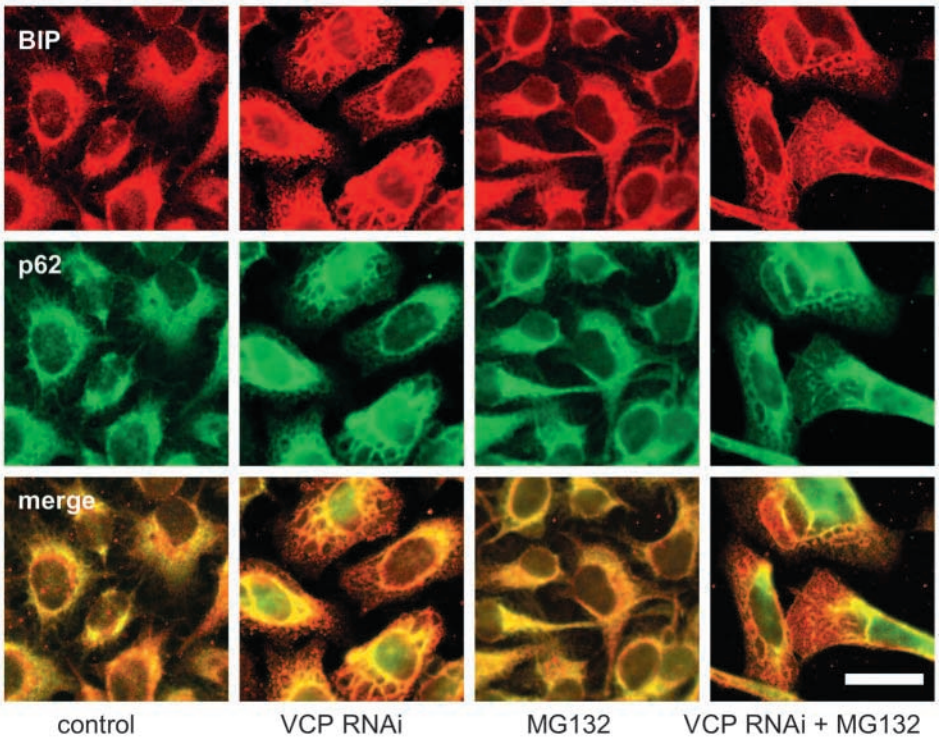

Fig. 6. RNAi of VCP alters the distribution of p62 and BIP. HeLa cells were subjected to RNAi of VCP for 4 days and/or treated with 10 μM MG132 for 6 hours. Immunofluorescence confocal microscopy was conduced for BIP (red) and for p62 (green). Scale bar, 20 μm.

Discussion

The results presented here demonstrate an important role for VCP in UPS function in insect and mammalian cells. RNAi of VCP in both *Drosophila* S2 cells and human HeLa cells caused significant accumulation of polyubiquitinated proteins, a characteristic of inhibited UPS function. The magnitude of the effect of VCP 'knock down' on UPS function is almost certainly underestimated in our experiments owing to incomplete reduction of VCP levels. Nevertheless, it is likely that VCP mediates degradation of only a subset of UPS substrates. Recent reports have demonstrated that VCP is required for multiple aspects of ERAD, including the selective degradation of mutant ER proteins and the constitutive and regulated degradation of normal ER proteins (Bays et al., 2001; Ye et al., 2001; Jarosch et al., 2002; Braun et al., 2002; Rabinovich et al., 2002). Thus, the effects of RNAi of VCP observed here probably include, but might not be limited to, constitutive ERAD. Previous work also has implicated VCP in the degradation of IκB (Dai et al., 1998) and cyclin B1 (Dai and Li, 2001), proteins with no obvious relationship to ERAD. Each of these proteins is selectively degraded while part of a multiprotein complex whose other components are spared. Such selective proteolysis might involve the ATP/ubiquitin-dependent chaperone or 'segregase' activity that VCP also uses

Table 3. Distribution of cell cycle phases in HeLa cells

	G1/G0 (%)	S (%)	G2/M (%)	DNA <2C (%)
Control	69.6	8.9	21.6	1.5
VCP RNAi	27.0*	22.3*	50.7*	22.7*
β5 RNAi	54.3*	10.9	34.8*	5.7*
MG132	57.2*	9.1	33.8*	2.0

HeLa cells were incubated for either 2 days or 4 days after transfection with siRNAs targeting degradation of mRNAs of the indicated proteins or treated for 24 hours with 10 μM MG132. 10,000 cells were counted for each sample. To calculate the proportion of cells within the cell cycle, only the cells with DNA content between 2C and 4C were considered.

*P<0.01 compared with control, calculated by χ^2 frequency tables.

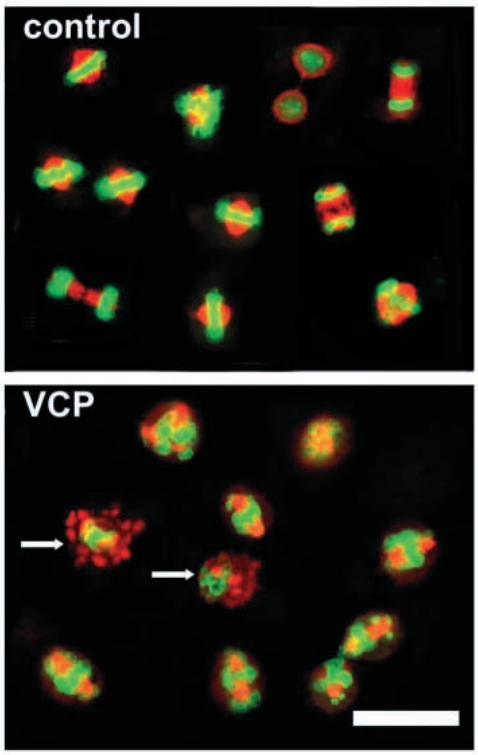

Fig. 7. RNAi of VCP causes mitotic abnormalities in HeLa cells. HeLa cells were subjected to RNAi of VCP for 2 days. Compound images of mitotic cells from control and RNAi cells were labelled with anti-α-tubulin (red) and the DNA dye Yo-Pro iodide (green). Scale bar, 20 μm.

for ERAD (Braun et al., 2002). Thus, VCP could exert general regulation of UPS function in instances where dissociation of multiprotein complexes is linked to proteolysis. We are currently attempting to identify those cellular proteins whose degradation is impaired in response to RNAi of VCP to assess the extent of VCP's role in intracellular protein degradation.

VCP has been implicated in a range of cellular functions (see Introduction). Multiple VCP-containing complexes could provide specificity and/or unique regulatory features required for given roles. For example, Ufd1 and Npl4 are required for VCP's role in ERAD (Bays et al., 2001; Braun et al., 2002). Our results show that RNAi of these proteins also caused significant accumulation of ubiquitinated proteins, thus supporting a role for the $VCP^{Ufd1\text{-}Npl4}$ complex in UPS function. By contrast, RNAi of p47 (a VCP-binding protein previously ascribed a role in membrane fusion) appears to play only a minor quantitative role in UPS function. In S2 cells, RNAi reduced *p47* mRNA to undetectable levels but had no demonstrable effect on accumulation of polyubiquitinated proteins. In HeLa cells, a >85% reduction of p47 protein by RNAi caused a small but significant accumulation of polyubiquitinated proteins. This effect was much less than that caused by a similar degree of reduction of VCP levels by RNAi. Thus, p47 appears to mediate the degradation of only a small subset of all VCP-dependent UPS substrates. These various results support the general hypothesis that different aspects of VCP function are mediated by different adaptor proteins with which it forms complexes. Our current incomplete analysis of these complexes by RNAi precludes direct comparisons of their relative quantitative contributions to the UPS function. Nevertheless, it is reasonable to suggest that individual complexes target different substrates, whose quantitative contributions to overall UPS-dependent proteolysis differ significantly. A goal of future work will be to identify specific substrates of the various VCP-containing complexes. VCP also could exert cellular effects via a general 'segregase' or chaperone activity not linked to proteolysis (Braun et al., 2002). While this manuscript was under review, two new VCP adaptor proteins were reported (Uchiyama et al., 2002; Nagahama et al., 2003). Additional work will be required to determine the roles of these proteins on UPS function and to distinguish between the proteolytic and non-proteolytic roles of VCP and its various binding proteins.

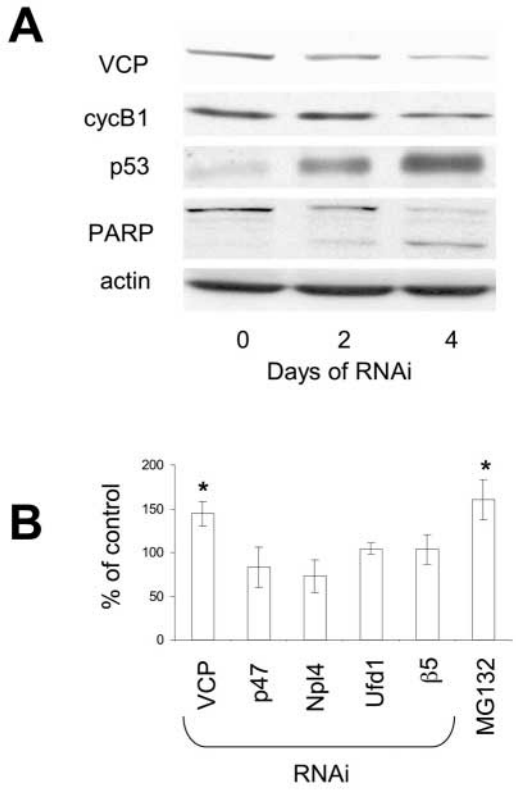

Fig. 8. RNAi of VCP induces apoptosis. (A) HeLa cells were subjected to RNAi of VCP for 2 days or 4 days. Equal amounts of protein from whole cell lysates were subjected to western blotting for the indicated proteins. (B) HeLa cells were subjected to RNAi for the indicated proteins for 4 days or treated with 5 μM MG132 for 6 hours. Caspase 3/7 activity was measured in each group of cells. Activity from control cells was assigned a value of 100% and activities from experimental groups are expressed as a percentage of the control. Results represent the mean (±s.e.m.) of three independent experiments. Each experimental group was compared to the control by Student's *t* test. The asterisk indicates $P<0.05$.

The role of VCP in multiple cellular processes might also be achieved by differential cellular subcellular localization. Previous reports have produced conflicting results regarding the cellular distribution of VCP. For example, microscopic studies have shown that VCP is distributed within both nucleus and cytoplasm but that the relative distribution within each compartment depended on the method of fixation and the exact antibody used (Peters et al., 1990; Madeo et al., 1998; Meyer et al., 2000). By contrast, biochemical studies have shown the VCP is enriched in the transitional ER (Zhang et al., 1994). In this study, we used immunofluorescence confocal microscopy to demonstrate that, in addition to a diffuse nuclear and cytoplasmic distribution, VCP was concentrated at the Golgi apparatus. Localization of VCP at the Golgi apparatus is consistent with its previously proposed roles in membrane fusion (Pleasure et al., 1993; Zhang et al., 1994; Acharya et al., 1995; Rabouille et al., 1995). The anti-VCP monoclonal antibody used in our studies recognized a single protein at the expected ~97 kDa in western blots of whole HeLa cell lysates, indicating that it was highly specific for VCP. Moreover, immunofluorescent labelling of HeLa cells was significantly reduced in cells subjected to RNAi of VCP, further supporting the specificity of the observed labelling pattern. In some cells, especially those subjected to RNAi of VCP, we found some immunoreactivity at discrete nuclear foci. Although the significance of this observation is unclear, it might be related to reports of VCP binding to various specific nuclear proteins (Zhang et al., 2001). In contrast to previous reports, we never observed VCP at centrosomes during mitosis (Madeo et al., 1998) or at aggresomes, which form around the centrosomes (Hirabayashi et al., 2001). These discrepancies might reflect the differences in cell types, antibodies and experimental conditions used in these various studies.

RNAi of VCP significantly reduced proliferation and promoted apoptosis of HeLa cells, indicating a crucial role for VCP in growth control. These effects might be mediated by p53, whose levels rose in response to RNAi of VCP. The UPS has a well-established role in controlling the cell cycle and apoptosis. For example, temporally regulated UPS-dependent proteolysis of multiple proteins is required for cell cycle progression in

most eukaryotic cells (Peters, 2002; Yew, 2001), and proteasome inhibitors block proliferation and induce apoptosis of rapidly growing mammalian cells (Wojcik, 2002). Our results suggest that VCP might mediate some of these effects. Proteasome inhibitors block the cell cycle at multiple points, causing the accumulation of mitotic HeLa cells characterized by condensed chromosomes arranged in metaphase plates (Wojcik et al., 1996a). RNAi of VCP also blocked the cell cycle and caused the accumulation of mitotic HeLa cells; these cells, however, were arrested at prometaphase/metaphase and had undercondensed chromatin and dispersed chromosomes. This effect might be related to the involvement of VCP in the degradation of cyclin B1 (Dai and Li, 2001). In yeast, VCP (Cdc48) is essential, identified originally as a cell-cycle mutant characterized by arrested cells with large buds and microtubules spread aberrantly throughout the cytoplasm from a single spindle plaque (Moir et al., 1982). Thus, VCP appears to be required for normal cell cycle progression, although the exact molecular mechanisms for this effect are unclear. Moreover, some previously reported disparities regarding the effects of VCP on growth and apoptosis are probably explained by different experimental design and systems. For example, RNAi of VCP had no effect on the growth of *Drosophila* S2 cells, but disruption of the VCP-encoding gene (*TER94*) blocked *Drosophila* germ-cell differentiation (Leon and McKearin, 1999). Overexpression of wild-type VCP blocked tumour-necrosis-factor-induced apoptosis in murine osteosarcoma cells but promoted apoptosis in both mammalian BAF-B03 pro-B cells and in the eyes of *Drosophila* (Shirogane et al., 1999; Asai et al., 2002; Hirabayashi et al., 2001; Higashiyama et al., 2002). Overexpression of VCP with loss-of-function mutations in the ATPase domain prevented neuronal cell apoptosis in *Drosophila* but promoted apoptosis in rat neuronal PC12 cells. These disparate results suggest that VCP function is determined by multiple and perhaps as-yet-undetermined factors, and might be specific for given cells and physiological conditions.

In contrast to RNAi of VCP, RNAi of Npl4, Ufd1 or p47 had little effect on cell growth. Although we cannot exclude the possibility that these distinctions result from differences in the degree of inhibited expression among the various proteins, we suggest that VCP exerts some effects independently of these adaptors or with other as-yet-unidentified adaptors. For example, another VCP-interacting protein, Ufd2, is cleaved by caspase during apoptosis and could therefore alter VCP function (Mahoney et al., 2002). Unfortunately, we have been unable to reduce Ufd2 levels by RNAi in HeLa cells (C.W., M.Y. and G.N.D., unpublished).

Ubiquitinated proteins that accumulate in cells as a consequence of impaired UPS function usually coalesce to form perinuclear, centrosome-associated aggregates, termed aggresomes (Wojcik et al., 1996b; Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999). Ubiquitinated proteins that accumulated in cells in response to RNAi of VCP did not form defined aggresomes but instead appeared as multiple small, dispersed cytoplasmic aggregates. This phenomenon was observed even in the presence of proteasome inhibitors, suggesting that VCP is required either directly for the formation of aggresomes or for a process whose inhibition indirectly disrupts the formation of aggresomes. In contrast to previous reports, we failed to detect VCP at centrosomes in untreated cells (Madeo et al., 1998) or at aggresomes in proteasome-inhibitor-treated cells (Hirabayashi et al., 2001), a discrepancy that might reflect multiple differences in experimental conditions.

Cells either treated with proteasome inhibitors or subjected to RNAi of VCP were also characterized by vacuolization of the cytoplasm; vacuolization was greatly accentuated in cells subjected to both treatments. These results are consistent with a phenotype previously observed in PC12 cells overexpressing a mutant form of VCP with defective ATPase function (Hirabayashi et al., 2001). The large vacuoles observed in our studies appear to represent a highly expanded ER. It seems unlikely that vacuole formation is a consequence of apoptosis because prevention of proteasome-inhibitor-induced apoptosis with caspase inhibitors had no effect on this process (Wagenknecht et al., 2000). Interestingly, RNAi of Ufd1, Npl4 and p47 did not induce vacuolization. It is also unlikely that vacuoles physically interfere with aggresome formation, because proteasome inhibitors often induce both aggresomes and vacuolization (Wojcik, 1997). Moreover, vacuolization induced by prolonged brefeldin-A treatment did not prevent aggresome formation caused by proteasome inhibitors (data not shown). Although these distinctions might be related to different but undocumented efficiencies of RNAi knock-downs among these proteins, they could also represent another example of distinct functions for different VCP-containing complexes. Additional detailed investigation of RNAi of VCP and these accessory proteins will be required to establish firmly their exact roles in vacuolization and whether these roles involve defective UPS.

In summary, our results demonstrate that VCP mediates the UPS-dependent degradation of a subset of cellular proteins. These proteins were not essential for normal growth of *Drosophila* S2 cells but were essential for normal growth of HeLa cells. Decreased VCP also promoted apoptosis of HeLa cells. Reduction of VCP levels induced vacuolization of the cytoplasm as a consequence of ER expansion, and inhibited formation of aggresomes. Although we have not evaluated possible non-proteolytic roles of VCP, we speculate that many of its functions might be linked to proteolysis. Our results provide general support for the role of VCP complexes as functional intermediates between the 26S proteasome and the ubiquitinated substrates it degrades. We are currently examining the molecular basis of this process for the various cellular features described here.

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