Ubiquilin recruits Eps15 into ubiquitin-rich cytoplasmic aggregates via a UIM-UBL interaction

Elsa Regan-Klapisz[1], Irina Sorokina[1], Jarno Voortman[1], Peter de Keizer[1], Rob C. Roovers[1], Peter Verheesen[1], Sylvie Urbé[2], Lara Fallon[3], Edward A. Fon[3], Arie Verkleij[1], Alexandre Benmerah[4] and Paul M. P. van Bergen en Henegouwen[1,*]

[1]Molecular Cell Biology, Institute of Biomembranes, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands
[2]Physiological Laboratory, University of Liverpool, Crown St, Liverpool, L69 3BX, UK
[3]Centre for Neuronal Survival, Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec, H3A 2B4, Canada
[4]Departement Maladies Infectieuses, Institut Cochin-U567 INSERM / UMR8104 CNRS, Pavillon G. Roussy, 27 rue de Fbg St Jacques, 75015 Paris, France

*Author for correspondence (e-mail: bergenp@bio.uu.nl)

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Summary

Eps15 and its related protein Eps15R are key components of the clathrin-mediated endocytic pathway. We searched for new binding partners of Eps15 using a yeast two-hybrid screen. We report here that ubiquilin (hPLIC1), a type-2 ubiquitin-like protein containing a ubiquitin-like domain (UBL) and a ubiquitin-associated domain (UBA), interacts with both Eps15 and Eps15R. Using glutathione-*S*-transferase pull-down experiments, we show that the first ubiquitin-interacting motif of Eps15 (UIM1) interacts directly with the UBL domain of ubiquilin, whereas it does not bind to ubiquitinated proteins. The second UIM of Eps15 (UIM2) binds poorly to the UBL domain but does bind to ubiquitinated proteins. Two other UIM-containing endocytic proteins, Hrs and Hbp, also interact with ubiquilin in a UIM-dependent manner, whereas epsin does not. Immunofluorescence analysis showed that endogenous Eps15 and Hrs, but not epsin, colocalize with green-fluorescent-protein-fused ubiquilin in cytoplasmic aggregates that are not endocytic compartments. We have characterized these green-fluorescent-protein-fused-ubiquilin aggregates as ubiquitin-rich intracytoplasmic inclusions that are recruited to aggresomes upon proteasome inhibition. Moreover, we show that endogenous Eps15 and endogenous ubiquilin colocalize to cytoplasmic aggregates and aggresomes. Finally, we show that the recruitment of Eps15 into ubiquilin-positive aggregates is UIM dependent. Altogether, our data identify ubiquilin as the first common UIM-binding partner of a subset of UIM-containing endocytic proteins. We propose that this UIM/UBL-based interaction is responsible for the sequestration of certain UIM-containing endocytic proteins into cytoplasmic ubiquitin-rich protein aggregates.

Key words: Eps15, Hrs, Ubiquilin, UIM, UBL, Aggresomes

Introduction

Eps15 [epidermal-growth-factor (EGF) receptor pathway substrate 15] and Eps15R (Eps15-related) are both essential components of the clathrin-mediated endocytic pathway. Eps15 interacts with the adaptor complex AP2 and is involved in the initial steps of clathrin-coated-pit formation (Benmerah et al., 1995; Morgan et al., 2003). Eps15 is composed of three structural domains: the N-terminal domain (domain I) contains three EH (Eps15 Homology) domains that interact with proteins containing tandem NPF (asparagine, proline, phenylalanine) repeats, such as epsin; the second domain (domain II) is a coiled-coil region involved in Eps15 oligomerization; and the C-terminal domain (domain III) contains binding sites to the α-subunit of the clathrin adaptor-protein complex AP-2 and two ubiquitin-interacting motifs (UIMs), located at the very C-terminal end of Eps15. The UIM is a conserved protein motif that was first identified in the S5a subunit of the proteasome (Hofmann and Falquet, 2001; Young et al., 1998) and was subsequently found in a range of proteins involved in ubiquitination, ubiquitin metabolism and endocytosis (Hofmann and Falquet, 2001). The endocytic proteins Hrs, epsin, STAM and STAM2/Hbp, and their yeast counterparts Vps27, ENT1/2 and HSE1 contain one to three UIMs and have been shown to bind ubiquitin and ubiquitinated proteins by way of their UIM (Bilodeau et al., 2002; Katz et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). In the case of Eps15, the second UIM acts as an ubiquitin recognition motif, whereas the first UIM is not able to bind ubiquitinated proteins (Polo et al., 2002). Importantly, both UIMs are essential for the ubiquitination of Eps15, suggesting that they contain docking sites for an E3 ubiquitin ligase, but they do not contain the site of ubiquitination (Klapisz et al., 2002; Polo et al., 2002). The ubiquitination signal activity of the UIM has also been characterized in epsin, Hrs and STAM/Hbp (Katz et al., 2002; Oldham et al., 2002; Polo et al., 2002).

The role of Eps15 ubiquitination and the function of its UIMs in endocytosis are still a matter of debate. Since the characterization of these UIMs, it has been suggested that Eps15 could recruit ubiquitinated receptors from the plasma membrane through its ubiquitin-binding ability (de Melker et al., 2004; Polo et al., 2002; Riezman, 2002). Recent data

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suggest that the UIM of Eps15 is necessary for the recruitment of the ubiquitinated EGF receptor (EGFR) into a non-clathrin internalization pathway (Sigismund et al., 2005). Moreover, Eps15 was found in complex with Hrs and STAM/Hbp, and was shown to localize with ubiquitinated proteins to endosomal membranes (Bache et al., 2003). Therefore, it was suggested that Eps15 participates in the endosomal sorting of ubiquitinated cargo proteins together with Hrs and STAM (Bache et al., 2003). However, more evidence is needed to validate this model. The role of the Hrs/Vps27 UIM is better documented. Both Hrs and Vps27 UIMs have been shown to be involved in the sorting of ubiquitinated cargoes into multivesicular bodies (Bilodeau et al., 2002; Raiborg et al., 2002; Shih et al., 2002).

The UIMs of diverse endocytic proteins have been shown to bind recombinant ubiquitin or ubiquitinated proteins from cellular lysates (Bilodeau et al., 2002; Katz et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Although Eps15 and epsin were shown to co-immunoprecipitate ubiquitinated EGFR in a UIM-dependent manner (Sigismund et al., 2005), there is no evidence of a direct interaction. Indeed, the UIM-containing endocytic proteins might interact with other ubiquitinated proteins present in the EGFR-containing complex. To date, no binding partner has been identified for the UIM of endocytic proteins.

In this work, we have identified ubiquilin, a type 2 ubiquitin-like protein (also known as PLIC-1) as the first characterized binding partner of the UIMs of the endocytic proteins Eps15, Hrs and Hbp. We show that Eps15 and Hrs colocalize and interact with ubiquilin. Furthermore, we provide evidence that the UIMs of Eps15 and Hrs interact in a direct manner with the ubiquitin-like domain (UBL) of ubiquilin. Ectopically expressed ubiquilin localizes to ubiquitin-rich cytoplasmic aggregates that form aggresomes upon proteasome inhibition. We show that endogenous Eps15 and Hrs are recruited by ubiquilin into these ubiquitin-positive cytoplasmic aggregates. In the case of Eps15, we show that this recruitment is UIM dependent. This is the first example of sequestration of UIM-containing endocytic proteins into ubiquitin-rich cytoplasmic aggregates.

Materials and Methods

Antibodies

The mouse monoclonal anti-FLAG antibody M5 was purchased from Sigma (St Louis, MO). The mouse monoclonal anti-Myc antibody clone 9E10, the mouse monoclonal antibodies against green fluorescent protein (GFP) (mixture of clone 7.1 and 13.1), the mouse monoclonal anti-His_6 antibody and the mouse monoclonal anti-haemagglutinin (anti-HA) antibody clone 12CA5 were obtained from Roche Diagnostics (Indianapolis, IN). The mouse monoclonal anti-ubiquitin antibody used for immunoblot analysis was obtained from StressGen Biotechnologies (Victoria, BC, Canada). The mouse monoclonal anti-ubiquitin antibody (clone FK2) used for immunofluorescence experiments was obtained from Affiniti Research (Exeter, UK). The rabbit polyclonal anti-Hrs and anti-Eps15 antibodies have been described previously (Sachse et al., 2002; Scott et al., 2002). The monoclonal mouse anti-CD63 antibody was a kind gift from E. Rubinstein (Rubinstein et al., 1996). Goat polyclonal antibodies against Epsin (R-20) and CALM (C-18) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-ubiquilin antibody was obtained from Affinity Bioreagents (Golden, CO). Cy3- and Alexa-labelled secondary antibodies were obtained from Molecular Probes (Leiden, The Netherlands).

Cell culture and transfection

Cos-1 cells and HeLa cells (ECACC, Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 7.5% and 10% foetal calf serum (Gibco), respectively, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere. For transient transfection, Cos-1 cells were seeded in 35 mm or 100 mm dishes [for immunoprecipitation or glutathione-*S*-transferase (GST) pull-down experiments, respectively], grown to 50% confluence and transfected with the indicated expression vectors using Fugene 6 reagent according to the manufacturer's instructions (Roche Diagnostic). Cells were harvested 24 hours after transfection. For immunofluorescence, subconfluent HeLa cells grown on coverslips were transfected using Fugene 6 reagent or using the calcium-phosphate transfection kit from Life Technologies. The cells were used 24 hours after transfection. MG132 or dimethylsulfoxide (DMSO) (vehicle) was added directly to the culture medium 8 hours after transfection and cells were incubated for 15-16 hours.

Plasmid constructs

The following plasmids have been described previously: pMT2SM-FLAG-Eps15; pMT2SM-FLAG-Eps15-UIM2mut (L883A/L885A); pcDNA3.1zeo-FLAG-Eps15; pcDNA3.1zeo-FLAG-Eps15-ΔI (deletion of amino acids 351-897), pcDNA3.1zeo-FLAG-Eps15-ΔII (deletion of amino acids 361-643), pcDNA3.1zeo-FLAG-Eps15-ΔIII (truncation at amino acid 648) and pcDNA3.1zeo-FLAG-Eps15-ΔUIM1+2 (truncation at amino acid 813); pcDNA3.1zeo-Myc-Eps15R (Klapisz et al., 2002); pEGFP-Hrs, pEGFP-Hrs-ΔUIM, pEGFP-Hrs-LSAA (Urbé et al., 2003); pS65T-GFP-CFTR (Moyer et al., 1998). The pcDNA3.1B plasmid (Chen et al., 1998), encoding His_6-tagged rat epsin 1, was a kind gift from H. Chen (Yale University, New Haven, CT). The pmiw-HA-Hbp construct (Hayakawa and Kitamura, 2000) was kindly provided by N. Kitamura (Tokyo Institute of Technology, Tokyo, Japan). The pmiw-Ha-Hbp-L176A/S177A (HA-Hbp-LSAA) and the pMT2SM-FLAG-Eps15-UIM1mut (E863A/S864A/E865A) vector were created using the QuickChange Site Directed Mutagenesis kit (Stratagene). The sequences of the primers are available on request. The pGBT8 vectors containing the cDNA encoding human Eps15 EH domains (amino acids 1-314) and human Eps15R EH domains (amino acids 1-351) were constructed by subcloning PCR fragments encoding the indicated sequences obtained from pGEX-5X1-hEps15 (Benmerah et al., 1995) and pBluescript-hEps15R (a kind gift from C. Schumacher, East Hanover, Novartis, NJ). The GST constructs encoding mouse Eps15 UIM1 (amino acids 847-877), UIM2 (amino acids 872-897) or UIM1+2 (amino acids 847-897) were obtained by subcloning PCR fragments into pGEX-5X-1. The GST constructs encoding mouse Hrs lacking the C-terminal region but containing the UIM (amino acids 1-454, GST-Hrs-ΔCT) or deleted from the UIM residues 257-278 (GST-Hrs-ΔCT-ΔUIM) were generated by subcloning into pGEX-4T-3 the *Bam*HI-*Xho*I fragments obtained from the previously described pGEMT-Hrs and pGEMT-Hrs-ΔUIM constructs (Urbé et al., 2003). The pEGFP-C2-HA-Ubiquilin vector was created by subcloning the cDNA encoding HA-tagged human ubiquilin (amino acids 14-589) from the pACT2 vector obtained in the yeast two-hybrid screen. The pGEX-5X-1-Ubiquilin vector was created by subcloning the cDNA of ubiquilin (amino acids 14-589) without the HA tag from pACT2-HA-Ubiquilin. The pEGFP-C2-UBL (ubiquitin-like domain of ubiquilin, amino acids 14-129) and the pEGFP-C2-UBA (ubiquitin-associated domain, amino acids 493-589) were obtained by subcloning PCR products into pEGFP-C2. The constructs encoding His_6-Myc-tagged ubiquilin (amino acids 14-589) or UBL (amino acids 14-129) were obtained by subcloning PCR

products into pUR5850 (Verheesen et al., 2003). All constructs were checked by DNA sequencing (Biolegio, Malden, The Netherlands).

Yeast two-hybrid screen

The bait construct pGBT8-Eps15-EH (amino acids 1-314 of human Eps15) was transformed into the *Saccharomyces cerevisiae* strain AH109 (BD-Clontech) using the lithium-acetate method. Subsequently, the human brain 'Matchmaker' cDNA library constructed in pACT2 (BD-Clontech) was transformed into AH109 and interacting clones were isolated on selective SC medium based on growth in the absence of tryptophan, leucine and adenine (SC –Trp –Leu –Ade) as previously described (de Graaf et al., 2004). False positives were eliminated based on their interaction with the negative control bait empty pGBT8 vector. The plasmids of the positive clones were isolated and sequenced (Eurogentec, Seraing, Belgium). The DNA sequence was compared with the GenBank/EMBL database using the BLAST program.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation was performed as previously described with some minor changes (Klapisz et al., 2002). For co-immunoprecipitation experiments, cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 2 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, supplemented with Complete™ protease-inhibitor cocktail (Roche Molecular Biochemicals). To check the ubiquitination of ubiquilin, cells were lysed in 'RIPA' buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, supplemented with Complete protease-inhibitor cocktail, and immunoprecipitation was performed as described previously (Klapisz et al., 2002). The data shown are representative of at least three independent experiments.

Protein purification and GST pull-down assay

The indicated His_6-Myc-tagged or GST fusion proteins were produced in *Escherichia coli* BL21-CodonPlus™-RIL (Stratagene) transformed with the appropriate plasmid. Protein expression was induced with 1 mM IPTG for 4 hours at 30°C. For GST fusion proteins, bacteria were lysed by sonication in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, supplemented with Complete protease-inhibitor cocktail, aliquoted in glycerol (final concentration 10%) and stored at –80°C. His_6-Myc-tagged proteins were purified by immobilized-metal-ion affinity chromatography (IMAC) using TALON beads (BD-Clontech) as described previously (Roovers et al., 1998). GST fusion proteins were purified from 1 ml bacterial-cell lysates with glutathione-agarose beads (Sigma) suspended in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P40, 2 mM $MgCl_2$ (pull-down buffer). After a 1 hour incubation, the beads were washed three times and resuspended in pull-down buffer supplemented with Complete protease-inhibitor cocktail. Transiently transfected cells were lysed in ice-cold pull-down buffer supplemented with Complete protease-inhibitor cocktail and clarified by centrifugation. The supernatant was mixed with the indicated GST fusion proteins coupled to glutathione-agarose beads for 1 hour at 4°C. For GST pull-down experiments using His_6-Myc-tagged recombinant proteins, 10 μg His_6-Myc-UBL or 30 μg His_6-Myc-ubiquilin were mixed with 10 μg of the indicated GST fusion proteins coupled to glutathione-agarose beads for 1 hour at 4°C. The beads were washed three times with pull-down buffer and boiled in reducing Laemmli sample buffer. Samples were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue to assess equal loading of the GST fusion proteins. The immunoblot analysis and stripping of the PVDF membrane were performed as described previously (Klapisz et al., 2002). The data shown are representative of at least three independent experiments.

Immunofluorescence

Immunofluorescence and transferrin staining were performed as described previously (Benmerah et al., 2000; Klapisz et al., 2002). Cells were fixed with 4% formaldehyde or ice-cold methanol for 30 minutes, where indicated, as described previously. The samples were examined under an epifluorescence microscope (Leica) attached to a cooled CCD camera (Micromax, Princeton Instruments). The pictures were taken using Metamorph and the final figures were obtained using the NIH Image and Adobe Photoshop programs. Colour figures were obtained from black and white images using Photoshop. The data shown are representative of at least three independent experiments.

Selection of nanobodies (llama VHH) against Eps15 by phage display

The cDNA encoding Eps15-EH1-3 (amino acids 1-314) was generated by PCR from pCEV-Eps15 and cloned in frame with glutathione-*S*-transferase in pGEX2T. The GST fusion protein GST-Eps15-EH1-3 was produced in the *E. coli* strain BL21-CodonPlus-RIL (Stratagene). A llama single-domain antibody fragment, also called a nanobody or VHH (variable heavy-chain region of the heavy-chain antibody), was selected against the three EH domains of Eps15 (Eps15-EH1-3) by phage display as described previously (Dolk et al., 2005). Briefly, a VHH anti-GST (10 $\mu g\ ml^{-1}$ in PBS) was coated onto a Maxisorp 96-well plate (Nunc) during an overnight incubation at 4°C. Non-specific binding was prevented by blocking with 4% skimmed milk in PBS (MPBS) at room temperature (RT) for 30 minutes before an excess of purified GST-Eps15-EH1-3 was added in 0.1% MPBS. After washing with PBS, 10^{11} library phages from a large naive library (kindly provided by Unilever Research, Vlaardingen, The Netherlands) were added and incubated in 2% MPBS, 1% bovine serum albumin for 90 minutes at RT. After extensive washing and elution with 100 mM triethylamine for 10 minutes at RT, phages were neutralized and multiplied according to standard procedures (Dolk et al., 2005). 10^9 phages in 2% MPBS, 1% bovine serum albumin were used for a second selection round against the directly immobilized GST-Eps15-EH1-3 coated onto a Maxisorp plate in a concentration of 10 $\mu g\ ml^{-1}$ in PBS. Single clones obtained after the second selection round were tested in an enzyme-linked immunosorbent assay for binding to GST-Eps15-EH1-3 using the Myc tag for detection. PCR products of the positive VHH single clones were analysed by *Hin*fI restriction pattern analysis. The cDNAs of the positive VHH clones were recloned into the vector pUR5850 (Verheesen et al., 2003) allowing the expression of a triple-tagged protein in the periplasmic space of *E. coli* containing c-Myc-, His_6- and biotinylation (LRSIFEAQKMEW) tags. The last of these tags induces the biotinylation upon expression in the *BirA*-gene-containing *E. coli* strain AVB101 (Avidity, Denver, CO) (Schatz, 1993). Llama VHH anti-Eps15 nanobodies were finally expressed in *E. coli* TG1 cells and purified by IMAC. The anti-Eps15 VHH clone used in this study was chosen from the other positive clones for its ability to detect Eps15 in immunoblot detection, immunoprecipitation and immunofluorescence. For immunofluorescence and western blotting, the llama VHH anti-Eps15 was detected with the mouse monoclonal anti-Myc antibody clone 9E10 followed by the appropriate secondary antibodies. The data shown are representative of at least three independent experiments.

Results

Eps15 and Eps15R interact with ubiquilin

In order to identify new binding partners of Eps15, we performed a yeast two-hybrid screening of a human brain

cDNA library using the first domain of human Eps15 as a bait (residues 1-314, containing three copies of the EH domain). From several interacting clones, we isolated two clones coding for nearly full-length ubiquilin (residues 14-589, GenBank accession number NM_013438). As shown in Fig. 1A, the interaction with the Eps15 EH domains was confirmed after retransformation in yeast. A bait encoding the EH domains of human Eps15R (residues 1-351) also interacted with ubiquilin (Fig. 1A). Ubiquilin is a multimodular protein composed of a UBL at its N-terminus, a central domain containing Asn-Pro repeats (including three NPF motifs) and a C-terminal UBA.

In order to confirm the interaction between ubiquilin and Eps15 or Eps15R, we expressed GFP-tagged ubiquilin (amino acids 14-589) and FLAG-tagged Eps15 or Myc-tagged Eps15R in Cos-1 cells and performed a coimmunoprecipitation assay. Fig. 1B shows that GFP-ubiquilin was coimmunoprecipitated with FLAG-Eps15 and with Myc-Eps15R. Surprisingly, GFP-ubiquilin in both cases displayed a ladder-like pattern in SDS-PAGE, reminiscent of the pattern of ubiquitinated proteins. These bands of higher molecular mass were not detected in the total lysates, suggesting that these modified forms of ubiquilin are enriched in a complex with Eps15. We examined the possibility that the GFP-ubiquilin laddering observed on SDS-PAGE could correspond to ubiquitinated GFP-ubiquilin. We immunoprecipitated GFP-ubiquilin in stringent conditions using RIPA buffer in order to get rid of potential ubiquitinated binding partners of ubiquilin (Donaldson et al., 2003). The anti-ubiquitin immunoblot showed a smear characteristic of polyubiquitinated proteins (Fig. 2A), indicating that GFP-ubiquilin could be ubiquitinated. We next examined whether the higher-molecular-mass species of GFP-ubiquilin that coimmunoprecipitate with Eps15 (Fig. 1B) were ubiquitinated forms of GFP-ubiquilin. FLAG-Eps15 and GFP-Ubiquilin were co-transfected in Cos-1 cells, FLAG-Eps15 was immunoprecipitated, and immunodetection was first performed with an anti-ubiquitin antibody. The anti-ubiquitin antibody detected a signal only when GFP-ubiquilin was coimmunoprecipitated with FLAG-Eps15 (Fig. 2B, right lane), whereas FLAG-Eps15 alone did not give any signal (Fig. 2B, left lane). This result suggests that ubiquitinated forms of GFP-ubiquilin are present in the Eps15 immunoprecipitate. We next examined whether ubiquitination of ubiquilin was required for the interaction with Eps15. A non-ubiquitinated bacterially expressed GST-ubiquilin was able to pull down FLAG-Eps15 from cell lysates (Fig. 2C), showing that ubiquitination of ubiquilin is not required for the interaction with Eps15.

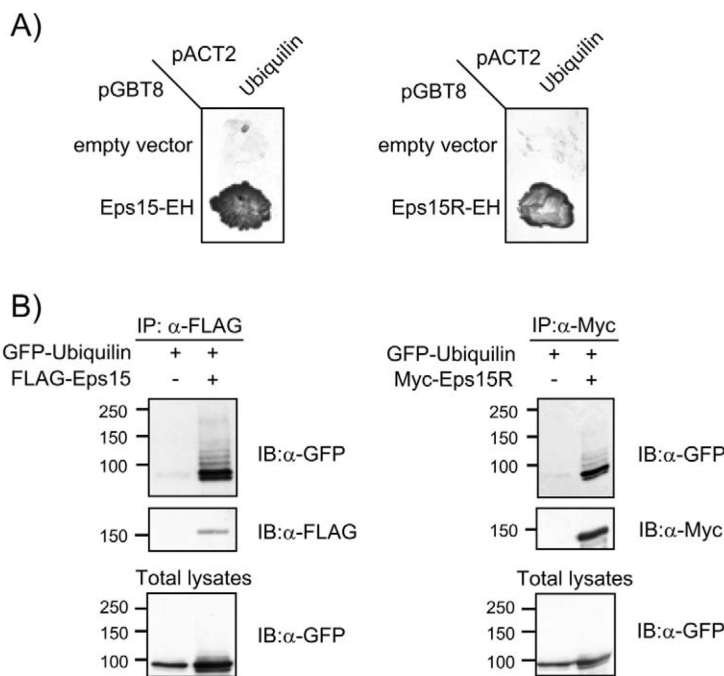

Fig. 1. Identification of an interaction between Eps15 and ubiquilin. (A) The pACT2 construct encoding ubiquilin (amino acids 14-589) fused to the GAL4 transactivation domain was co-transformed with pGBT8 constructs expressing the GAL4 DNA binding domain alone (empty vector) or fused with the EH domains of Eps15 or Eps15R (Eps15-EH or Eps15R-EH) in *S. cerevisiae* strain AH109. Yeast was grown on selective medium (SC –Trp –Leu –Ade) for 4 days. (B) Cos-1 cells were transiently transfected with constructs encoding GFP-ubiquilin and FLAG-Eps15, Myc-Eps15R or empty vector (–), as indicated. Immunoprecipitation (IP) was performed with anti-FLAG (α-FLAG) or anti-Myc (α-Myc) antibody. The immunoprecipitates were separated by SDS-PAGE followed by immunoblotting (IB) with anti-GFP antibody (α-GFP). The membranes were stripped and reprobed with either anti-FLAG or anti-Myc antibody. Total lysates immunoblotted with anti-GFP antibody are shown as a control for GFP-ubiquilin expression. Notice the ladder-like pattern of ubiquilin in the immunoprecipitates but not in the total lysates.

UIM domains of Eps15 interact with the UBL domain of ubiquilin

In order to understand the structural requirements for the interaction between Eps15 and ubiquilin, we determined the domains involved in the interaction. Because Eps15 and ubiquilin are both multimodular proteins, we envisaged that their interaction might involve several protein-protein interaction domains. We expected from the yeast two-hybrid data that the EH domains of Eps15 might interact with the NPF motifs present in ubiquilin. Furthermore, the UBA domain of ubiquilin might bind to the ubiquitin moiety appended to Eps15. Also, the UBL domain of ubiquilin, which shares 35% identity with ubiquitin, was predicted to bind to a UIM of Eps15.

We first performed a coimmunoprecipitation experiment using GFP-ubiquilin and deletion mutants of Eps15 lacking one of its three structural domains (Fig. 3A). Surprisingly, deletion of the second domain (the coiled-coil region) and deletion of the third domain of Eps15 disrupted the interaction with ubiquilin, whereas deletion of the three EH domains (domain I) did not affect the interaction with ubiquilin (Fig. 3B). This latest result suggested that the EH domains of Eps15 are not essential for this interaction. To further examine this hypothesis, we used GST fused to the three EH domains (amino acids 1-314) in a GST pull-down assay with total lysates of Cos-1 cells overexpressing ubiquilin. We could not detect any interaction of the EH domains with ubiquilin in this assay (data not shown). Together, our results suggest that, although the EH domains of Eps15 are sufficient to promote an interaction with ubiquilin (detected in a yeast two-hybrid assay), this interaction might be of low affinity (not detected in a GST pull-down assay) and not essential.

Next, we examined the possibility that the loss of

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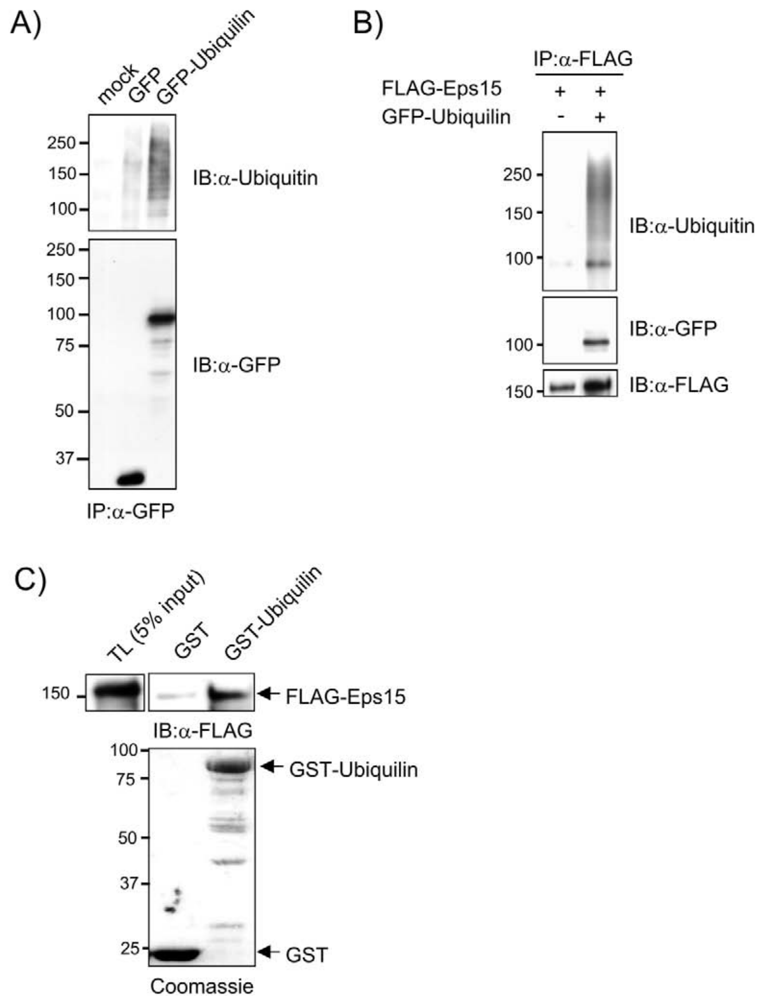

Fig. 2. Ubiquitinated GFP-ubiquilin interacts with Eps15 but ubiquitination is not essential for this interaction. (A) Cos-1 cells were transiently transfected with a vector encoding GFP-ubiquilin or GFP alone, or mock transfected. Cell extracts were subjected to immunoprecipitation (IP) with anti-GFP antibody (α-GFP) in RIPA buffer. Immunoprecipitates were separated by SDS-PAGE and immunoblotted (IB) with anti-ubiquitin antibody (α-ubiquitin). The membrane was stripped and reprobed with anti-GFP antibody. (B) Cos-1 cells were transiently co-transfected with FLAG-Eps15 and GFP-ubiquilin or empty vector (–), as indicated. Immunoprecipitation (IP) was performed with anti-FLAG antibody (α-FLAG). The immunoprecipitates were separated by SDS-PAGE followed by immunoblotting (IB) with anti-ubiquitin antibody. The membrane was stripped and reprobed with anti-GFP antibody. After a second stripping, the membrane was reprobed with anti-FLAG antibody. (C) Equal amounts of Cos-1 total cell lysate expressing FLAG-Eps15 (TL; 5% of the total input is shown) was incubated with GST or GST-ubiquilin immobilized on glutathione-Sepharose beads. The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-FLAG antibody. The lower panel shows a Coomassie staining of the membrane. The positions of GST and GST-ubiquilin are indicated.

interaction of ubiquilin with the Eps15 ΔIII mutant was due to the deletion of the UIMs. We therefore analysed the interaction of ubiquilin with Eps15 mutants carrying point mutations in either UIM. We used the previously described UIM2 mutant FLAG-Eps15-UIM2mut carrying the double mutation L883A/L885A and we generated a mutant of UIM1 by mutating the essential serine 864 and the two surroundings glutamate residues to alanine in the context of the full-length Eps15 (FLAG-Eps15-UIM1mut carrying the triple mutation E863A/S864A/E865A). Mutation of UIM1 resulted in a loss of ubiquitination as assessed by a previously described in vivo ubiquitination assay (Klapisz et al., 2002), confirming that the mutation had successfully disrupted the ubiquitination signal activity of UIM1 (data not shown). As shown in Fig. 4A, mutation of either UIM1 or UIM2 of Eps15 disrupted the interaction with ubiquilin. The loss of interaction between ubiquilin and the Eps15 UIM mutants could be attributed either to the loss of ubiquitination of Eps15 (caused by the disruption of the ubiquitination signal carried by either UIMs) or to the loss of a protein-protein

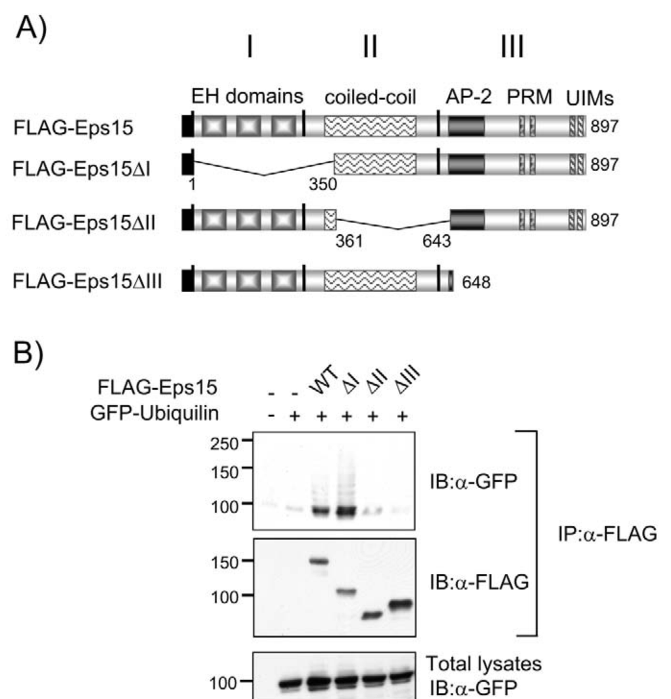

Fig. 3. The coiled-coil domain and the third domain of Eps15 are necessary for the interaction with ubiquilin. (A) The FLAG-tagged Eps15 deletion mutants. The three structural domains of Eps15 (I, II and III) were deleted as indicated. The FLAG tag (black box) is located at the N-terminus of the constructs. AP-2, AP-2 binding sites; PRM, proline-rich motif; UIMs, ubiquitin-interacting motifs. (B) Cos-1 cells were transiently co-transfected with GFP-ubiquilin and the indicated FLAG-Eps15 constructs (WT, wild type) or empty vector (–). Immunoprecipitation (IP) was performed with anti-FLAG antibody (α-FLAG) and samples were subjected to immunoblotting with anti-GFP antibody (α-GFP). The membrane was stripped and reprobed with anti-FLAG antibody. Notice that the FLAG-Eps15 constructs are expressed at similar levels. (bottom) Total cell lysates immunoblotted with anti-GFP antibody as a control for GFP-ubiquilin expression.

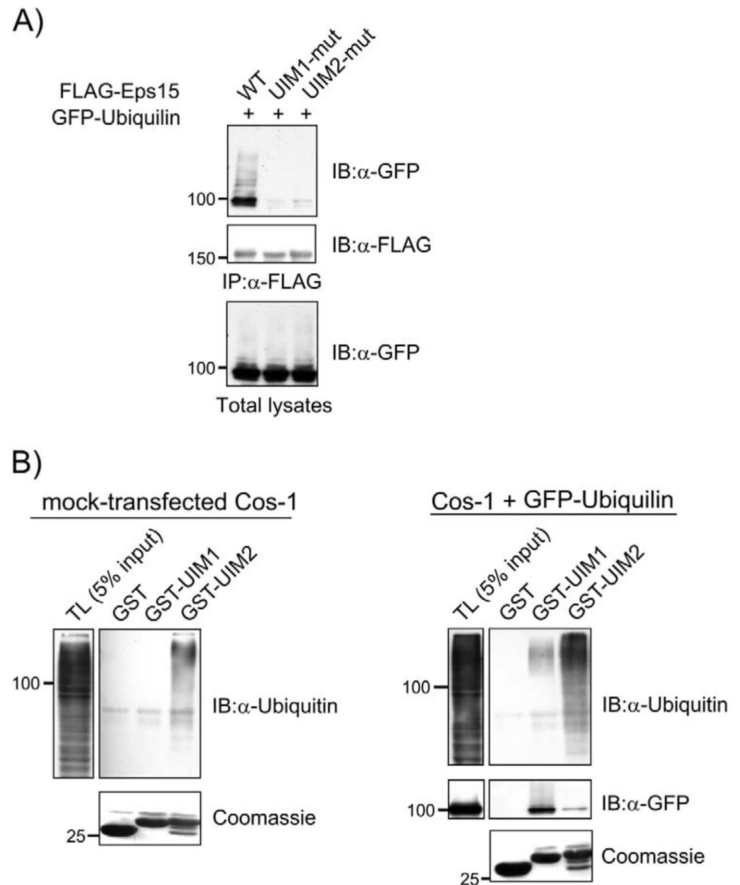

Fig. 4. Interaction between the UIMs of Eps15 and Ubiquilin. (A) Cos-1 cells were transiently transfected with GFP-ubiquilin and FLAG-Eps15 wild-type (WT), UIM1mut (E863A/S864A/E865A) or UIM2mut (L883A/L885A). Immunoprecipitation (IP) was performed with anti-FLAG antibody (α-FLAG). The immunoprecipitates were subjected to immunoblotting (IB) with anti-GFP antibody (α-GFP). The membrane was stripped and reprobed with anti-FLAG antibody. Notice that equal amounts of Eps15 were present in each immunoprecipitate. (bottom) An anti-GFP immunoblot of total cell lysates as a control for GFP-ubiquilin expression. (B) Equal amounts of GST alone, GST-UIM1 (amino acids 847-877 of mEps15) or GST-UIM2 (amino acids 872-897 of mEps15) immobilized on glutathione-Sepharose beads was incubated with total cell lysates (TL, 5% of the total input is shown) of mock-transfected or GFP-ubiquilin transfected Cos-1 cells (Cos-1 + GFP-ubiquilin). The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-ubiquitin antibody (α-ubiquitin). In the experiment using GFP/ubiquilin-transfected cells, the membrane was stripped and reprobed with anti-GFP antibody. Notice that the non-ubiquitinated GFP-ubiquilin (100 kDa) was precipitated by GST-UIM1 and, to a lesser extent, by GST-UIM2. (bottom) Coomassie staining of the membranes.

interaction motif. In order to distinguish between these two possibilities, GST fusion proteins encoding UIM1 (amino acids 847-877) or UIM2 (amino acids 872-897) of Eps15 were used in a pull-down assay with lysates of Cos-1 cells transfected or not with GFP-ubiquilin. Surprisingly, UIM1 was able to bind ubiquilin (Fig. 4B, right) but was not able to bind ubiquitinated proteins (Fig. 4B, left). By contrast, the UIM2 of Eps15 was able to bind ubiquitinated proteins (Fig. 4B, left) but it bound ubiquilin much less well than UIM1 (Fig. 4B, right). Together these data indicate that the UIMs of Eps15 are involved in a direct protein-protein interaction with ubiquilin.

The ability of the UIMs of Eps15 to interact with ubiquilin prompted us to test whether this interaction was mediated via the UBL domain of ubiquilin. We created GFP-tagged constructs encoding either the UBL domain (amino acids 14-129) or the UBA domain (amino acids 493-589) (Fig. 5A). These constructs were expressed with FLAG-Eps15 and immunoprecipitated with anti-GFP antibody. FLAG-Eps15 was coimmunoprecipitated with the UBL domain but not with the UBA domain, although both constructs were expressed at similar levels (Fig. 5B). Moreover, the UBL domain was coimmunoprecipitated with endogenous Eps15 (data not shown). These results show that the UBL domain of ubiquilin is sufficient to promote an interaction with full-length Eps15. We next examined whether the UIMs of Eps15 were able to bind directly to the UBL domain of ubiquilin. Pull-down experiments were performed using GST-UIM constructs and purified bacterially expressed His_6-Myc-tagged ubiquilin or His_6-Myc-UBL. Fig. 5C shows that both recombinant His_6-Myc-Ubiquilin and His_6-Myc-UBL were precipitated by GST-UIM1. Interestingly, GST-UIM2 bound to the recombinant ubiquilin and UBL much less well than GST-UIM1, confirming the data obtained by pulling down GFP-ubiquilin from Cos-1 cell lysates (Fig. 4B, right). Altogether, these data show that there is a direct interaction between the UIMs of Eps15 and the UBL domain of ubiquilin and that the UIM1 of Eps15 interacts more efficiently with the UBL domain than the UIM2.

Hrs and Hbp interact with ubiquilin in a UIM-dependent manner

The UIM motif was identified in several endocytic proteins in which it was shown to act both as ubiquitination signal and as ubiquitin recognition signal. We investigated the possibility that ubiquilin is a general UIM-binding protein. We first tested the ability of ubiquilin to interact with two other UIM-containing endocytic proteins, Hrs and epsin. Hrs contains one UIM, whereas epsin1 contains three (Hofmann and Falquet, 2001). The GST-ubiquilin fusion protein was used to pull down lysates of cells transfected with either GFP-tagged Hrs or His_6-tagged epsin1. As shown in Fig. 6A, only Hrs could be found in GST-ubiquilin precipitates, whereas epsin could not be detected under the same conditions. We next examined whether the interaction of Hrs with ubiquilin was dependent on its UIM, as is the case for Eps15. To test this hypothesis, Hrs mutants

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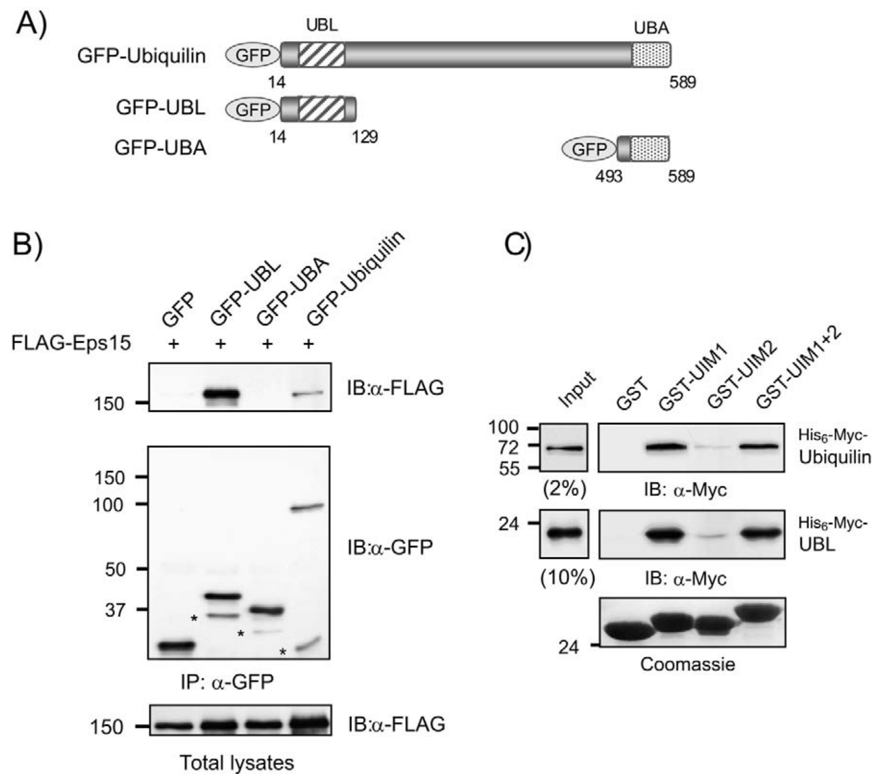

Fig. 5. The UBL domain of ubiquilin is sufficient to promote the interaction with Eps15 UIMs. (A) GFP-tagged ubiquilin constructs. UBA, ubiquitin-associated domain (amino acids 493-589); UBL, ubiquitin-like domain (amino acids 14-129). (B) Cos-1 cells were transfected with FLAG-Eps15 and GFP alone, GFP-ubiquilin, GFP-UBL or GFP-UBA. Cell lysates were subjected to immunoprecipitation (IP) with anti-GFP antibody (α-GFP) followed by immunoblotting (IB) with anti-FLAG antibody (α-FLAG). The membrane was stripped and reprobed with anti-GFP antibody to check the expression level of each GFP construct. Degradation products of the GFP-UBL, GFP-UBA and GFP-ubiquilin are marked with asterisks (*). (bottom) Anti-FLAG immunoblot of total cell lysates as a control for FLAG-Eps15 expression. (C) Equal amount of GST alone, GST-UIM1, GST-UIM2 or GST-UIM1+2 immobilized on glutathione-Sepharose beads was incubated with purified recombinant His_6-Myc-Ubiquilin or His_6-Myc-UBL (2% and 10%, respectively, of the total input are shown). The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-Myc antibody. (bottom) Representative Coomassie staining of the membrane for both experiments. Notice that both His_6-Myc-Ubiquilin and His_6-Myc-UBL were precipitated more efficiently by GST-UIM1 than by GST-UIM2.

carrying either a deletion of the UIM (GFP-Hrs-ΔUIM) or a mutation of the two essential UIM residues L269 and S270 to alanine (GFP-Hrs-LSAA) were tested in the GST-ubiquilin pull-down assay. Both the deletion and the point mutation of the unique UIM of Hrs completely abolished the precipitation of the Hrs constructs by GST-ubiquilin (Fig. 6B), indicating that the UIM of Hrs is required for the interaction with ubiquilin. The Hrs-binding protein Hbp (also known as STAM2) was also tested for its ability to interact with ubiquilin. As shown in Fig. 6C, HA-Hbp was precipitated by GST-ubiquilin. We next tested whether this interaction was dependent on the unique UIM of Hbp by using an Hbp mutant carrying a mutation of the two essential UIM residues L176 and S177 to alanine (HA-Hbp-LSAA). Fig. 6C shows that the mutation of the unique UIM of Hbp disrupted the interaction with ubiquilin. Altogether, our data show that ubiquilin interacts with Eps15, Hrs and Hbp in a UIM-dependent manner.

We next examined the involvement of the UBL domain of ubiquilin in the UIM-dependent binding of Hrs. In a similar way to a GST construct encoding the two UIMs of Eps15 (amino acids 847-897), a GST-Hrs construct lacking the C-terminal region but containing the UIM (amino acids 1-454, GST-Hrs-ΔCT) was able to precipitate the UBL domain of ubiquilin from GFP-UBL transfected cell lysates. By contrast, the interaction with the UBL domain was lost when the UIM was further deleted from the GST-Hrs-ΔCT construct (GST-Hrs-ΔCT-ΔUIM), showing that the UIM is required for this interaction (Fig. 6D). Finally, the GST-Hrs-ΔCT construct was able to pull down purified bacterially expressed His_6-Myc-ubiquilin and His_6-Myc-UBL, whereas the interaction was lost with the GST-Hrs-ΔCT-ΔUIM construct (data not shown). Altogether, these results show that, like the UIMs of Eps15, the UIM of Hrs interacts directly with the UBL domain of ubiquilin, suggesting that the UBL-UIM interaction is conserved among certain members of the family of UIM-containing endocytic proteins.

Hrs and Eps15 localize with ubiquilin to cytoplasmic aggregates

To further confirm that Eps15 and Hrs functionally interact with ubiquilin in vivo, their colocalization was tested by immunofluorescence microscopy. The GFP-ubiquilin construct was used to analyse the localization of ubiquilin. In HeLa cells (Fig. 7Aa,c,e) and Cos-1 cells (data not shown), GFP-ubiquilin showed a punctate aggregate-like pattern dispersed throughout the cytoplasm, in addition to a diffuse cytoplasmic staining. The ubiquilin-aggregate-like structures were also observed when we used a HA-ubiquilin construct, although their number and size were reduced (data not shown). These ubiquilin-positive puncta have been previously described in HeLa cells for endogenous ubiquilin and for overexpressed ubiquilin constructs (GFP- and Myc-tagged as well as untagged ubiquilin) (Mah et al., 2000). The staining of GFP-UBL (amino acids 14-129) and GFP-UBA (amino acids 493-589) was similar to that of GFP alone (data not shown), indicating that the full-length GFP-ubiquilin is required for localization in the punctate structures. Endogenous Eps15 (Fig. 7Aa,b) and endogenous Hrs (Fig. 7Ae,f), but not endogenous epsin (Fig. 7Ac,d), clearly colocalized with GFP-ubiquilin to these punctate structures. These data confirm that ubiquilin interacts

in vivo with Eps15 and Hrs but not with epsin, in agreement with our previous results obtained by biochemical approaches.

Eps15 is a constitutive, ubiquitous component of clathrin-coated pits (CCPs) and is associated with endosomes upon massive EGF stimulation (Sorkina et al., 1999; Torrisi et al., 1999) or after stabilization of the endosomal clathrin coat (Bache et al., 2003; Raiborg et al., 2001). Hrs is localized to early endosomes, where it participates in the sorting of ubiquitinated growth factor receptors into the lysosomal degradation pathway (Raiborg et al., 2002; Sachse et al., 2002; Urbé et al., 2003). GFP-ubiquilin did not localize to CCPs, as shown by the lack of colocalization with the CCP marker CALM (Fig. 7Ba), or with clathrin or AP-2 (data not shown). In addition, GFP-ubiquilin did not colocalize with early or with late endosomal markers such as transferrin (Fig. 7Bb) or CD63 (Fig. 7Bc), respectively, suggesting that the GFP-ubiquilin-positive puncta are not endosomes. Altogether, these results suggest that ubiquilin colocalizes with Eps15 and Hrs to cytoplasmic puncta that do not correspond to endocytic compartments.

Ubiquilin localizes to cytoplasmic aggregates that form aggresomes upon proteasome inhibition

We next tried to determine the nature of the cytoplasmic ubiquilin aggregates in which endogenous Eps15 and Hrs are sequestered upon GFP-ubiquilin expression. It was recently reported (Massey et al., 2004) that overexpressed ubiquilin was present in ubiquitin-rich structures that contained overexpressed presenilin-2 (PS2) and that it localized to PS2-containing aggresomes upon proteasome inhibition. In order to determine whether the GFP-ubiquilin cytoplasmic aggregates might form aggresomes, we studied the cellular distribution of GFP-ubiquilin in HeLa cells treated overnight with the proteasome inhibitor MG132. Two structures have been described for the aggresome: a single spherical aggresome (1-

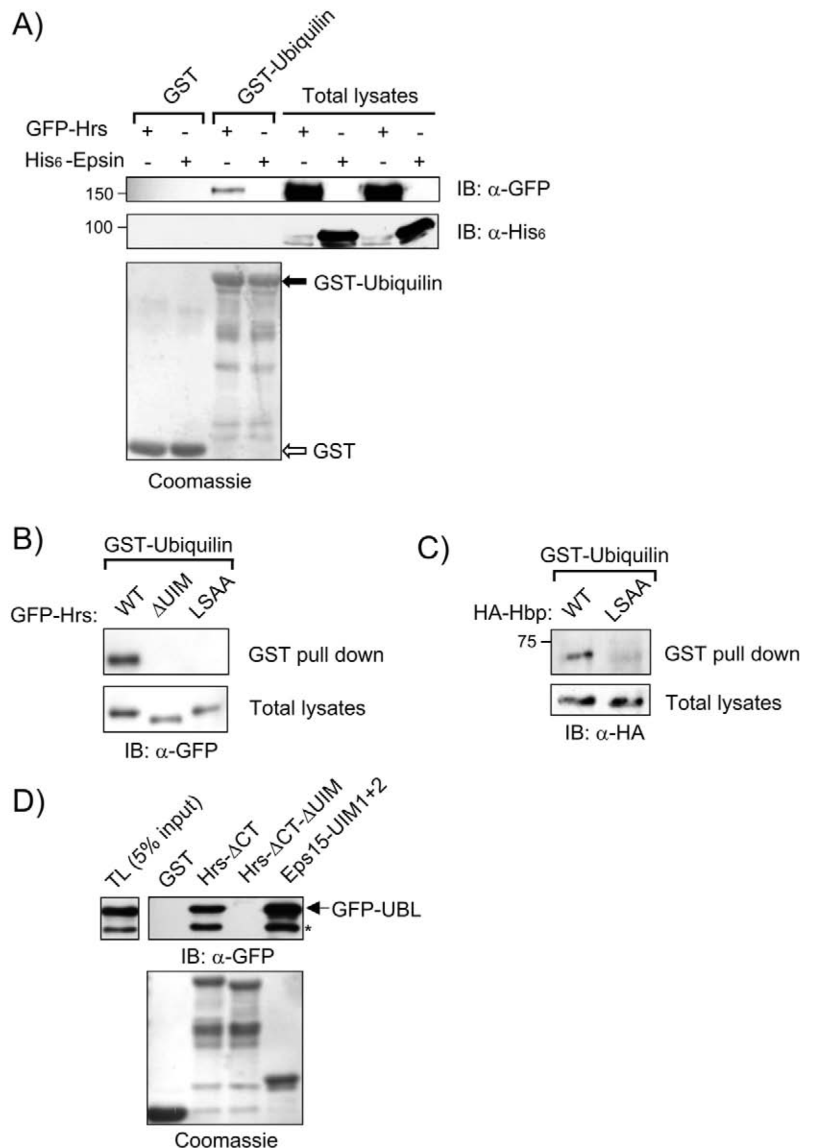

Fig. 6. Hrs and Hbp interact with ubiquilin in a UIM-dependent manner, whereas epsin does not interact with ubiquilin. (A) Equal amounts of GST or GST-ubiquilin immobilized on glutathione-Sepharose beads were incubated with total cell lysates of Cos-1 cells transfected with GFP-Hrs or His_6-epsin (5% of the initial input is shown). The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-ubiquitin anti-GFP antibody (α-GFP). The membrane was stripped and reprobed with anti-His_6 antibody (α-His_6). (bottom) Coomassie staining of the membrane. The positions of GST and GST-ubiquilin are indicated. (B) GST-ubiquilin immobilized on glutathione-Sepharose beads was incubated with total cell lysates of Cos-1 cells transfected with GFP-Hrs wild type (WT), GFP-Hrs-ΔUIM or GFP-Hrs-LSAA in which the two essential UIM residues L269 and S270 are mutated to alanine. The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-GFP antibody (top, GST pull down). (bottom) Anti-GFP immunoblot of total cell lysates (5% of the initial input) showing similar expression levels of the GFP-Hrs constructs. (C) GST-ubiquilin immobilized on glutathione-Sepharose beads was incubated with total cell lysates of Cos-1 cells transfected with HA-Hbp wild type (WT) or HA-Hbp-LSAA in which the two essential UIM residues L176 and S177 are mutated to alanine. The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-HA (top, GST pull down). (bottom) Anti-HA immunoblot of total cell lysates (5% of the initial input) showing similar expression levels of the HA-Hbp constructs. GST alone did not bind the HA-Hbp constructs (not shown). (D) Equal amounts of GST, GST-Hrs-ΔCT (amino acids 1-454), GST-Hrs-ΔCT-ΔUIM or GST-Eps15-UIM 1+2 (amino acids 847-897) was incubated with total cell lysates of Cos-1 cells transfected with GFP-UBL. The beads were washed and subjected to SDS-PAGE followed by immunoblotting (IB) with anti-GFP antibody. The pulled-down GFP-UBL is indicated with an arrow. The asterisk (*) indicates a degradation product of the UBL. (bottom) Coomassie staining of the membrane. The GST-Hrs-ΔCT and GST-Hrs-ΔCT-ΔUIM (at the top of the membrane) displayed several degradation or incompletely translated products.

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3 μm diameter) or an extended ribbon (Garcia-Mata et al., 2002). Fig. 8b shows that, upon proteasome inhibition, GFP-ubiquilin was localized to a perinuclear structure similar to a ribbon-type aggresome. Enrichment in ubiquitinated proteins is one of the main features of aggresomes (Garcia-Mata et al., 2002; Kopito, 2000). To assess whether the cytoplasmic aggregates formed by GFP-ubiquilin contain ubiquitinated proteins, we stained HeLa cells expressing GFP-ubiquilin with the FK2 anti-ubiquitin antibody that recognizes mono- and polyubiquitinated proteins, but not free ubiquitin. The cytoplasmic aggregates formed by GFP-ubiquilin stained positive for ubiquitin (Fig. 8c,d). Furthermore, when the cells were treated overnight with MG132, the aggresome-like structure formed by GFP-ubiquilin was strongly positive for ubiquitinated proteins (Fig. 8e,f).

In order to demonstrate positively that ubiquilin is sequestered in aggresomes upon proteasome inhibition, we made use of cystic fibrosis transmembrane regulator (CFTR; the first protein described to form aggresomes) (Johnston et al., 1998) to induce aggresome formation and followed ubiquilin localization. We used conditions similar to those described by Johnston et al. (Johnston et al., 1998) to induce the formation of CFTR-containing aggresomes. Cells co-transfected with HA-ubiquilin and GFP-CFTR were subjected to overnight treatment with MG132. This condition led to the formation of a CFTR-containing spherical aggresome (Fig. 8h) that also contained HA-ubiquilin (Fig. 8g).

We next examined whether Eps15 was found in ubiquilin-containing aggresomes. Cells co-transfected with GFP-ubiquilin and FLAG-Eps15 were subjected to overnight treatment with MG132. This condition led to the formation of aggresomes that contained both GFP-ubiquilin and FLAG-Eps15 (Fig. 9A). In order to ensure that the colocalization of Eps15 and ubiquilin in cytoplasmic aggregates and in aggresomes is not an epiphenomenon caused by overexpression, we investigated the colocalization of endogenous ubiquilin and endogenous Eps15 in mock-treated (0.1% DMSO) and MG132-treated cells. None of the available anti-Eps15 antibodies we tried could detect endogenous Eps15 in aggresomes. This could be caused by the low penetration capacity of conventional antibodies into the inner core of the aggresomes. Therefore, we decided to use a llama single-domain antibody fragment (VHH or nanobody) against Eps15. In addition to their extreme stability, llama VHH antibody fragments are the smallest intact antigen-binding fragments known (Dolk et al., 2005; Verheesen et al., 2003) and have better penetrating capacities into dense structures (Stijlemans et al., 2004). We selected a nanobody against domain I of Eps15, which contains the three EH domains. As shown in Fig. 9B, the anti-Esp15 nanobody specifically recognizes full-length FLAG-Eps15 but not a construct lacking the EH domains (FLAG-Eps15-ΔI). Moreover, it can immunoprecipitate endogenous Eps15 (Fig. 9C). We next used the anti-Eps15 nanobody to study the colocalization of endogenous Eps15 and endogenous ubiquilin. In mock-treated cells, the colocalization of endogenous ubiquilin and

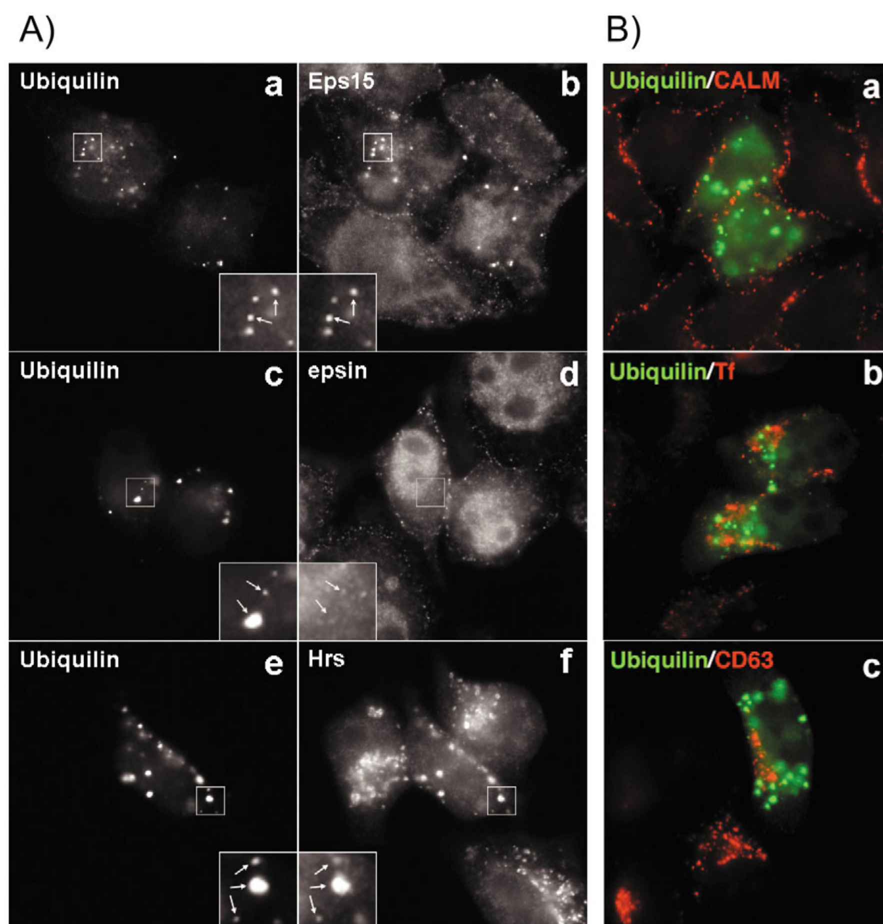

Fig. 7. Ubiquilin colocalizes with Eps15 and Hrs, but not with epsin, and it is not found in clathrin-coated pits or in endosomes. (A) Colocalization of GFP-ubiquilin with endogenous Eps15 and Hrs but not with epsin. HeLa cells transiently transfected with the GFP-ubiquilin construct (a-f) were processed for fluorescence microscopy using the rabbit anti-Eps15 (a,b) and anti-Hrs (e,f) polyclonal antibodies, and the goat anti-epsin (c,d) polyclonal antibody. The first antibodies were revealed by Alexa-594-labelled goat anti-rabbit and donkey anti-goat immunoglobulin secondary antibodies. (a,c,e) Green fluorescence emitted by GFP. (b,d,f) Red fluorescence emitted by Alexa 594. Insets show higher magnifications of representative areas in which arrows stress GFP-ubiquilin dots. (B) Ubiquilin is found in neither CCPs nor endosomes. HeLa cells transiently transfected with the GFP/ubiquilin-encoding construct (a-c) were processed for fluorescence microscopy using the goat anti-CALM polyclonal antibody (a) and the mouse anti-CD63 monoclonal antibody (c) revealed with Alexa-594-labelled donkey anti-goat and goat anti-mouse immunoglobulins secondary antibodies, respectively. For transferrin staining (b), the GFP/ubiquilin-transfected cells were allowed to internalize Alexa-594-labelled transferrin for 30 minutes. GFP staining and Alexa-594 staining are shown in green and red, respectively.

endogenous Eps15 in cytoplasmic aggregates was occasionally observed in big aggregates (Fig. 9D, top). When cells were treated overnight with MG132 to induce aggresome formation, endogenous ubiquilin and endogenous Eps15 were both localized to aggresomes.

In conclusion, our data demonstrate that the GFP-ubiquilin aggregates are ubiquitin-rich intracytoplasmic inclusions that are recruited to aggresomes upon proteasome inhibition. Moreover, both ectopically expressed and endogenous Eps15 and ubiquilin are localized to these cytoplasmic aggregates and are recruited to aggresomes upon proteasome inhibition.

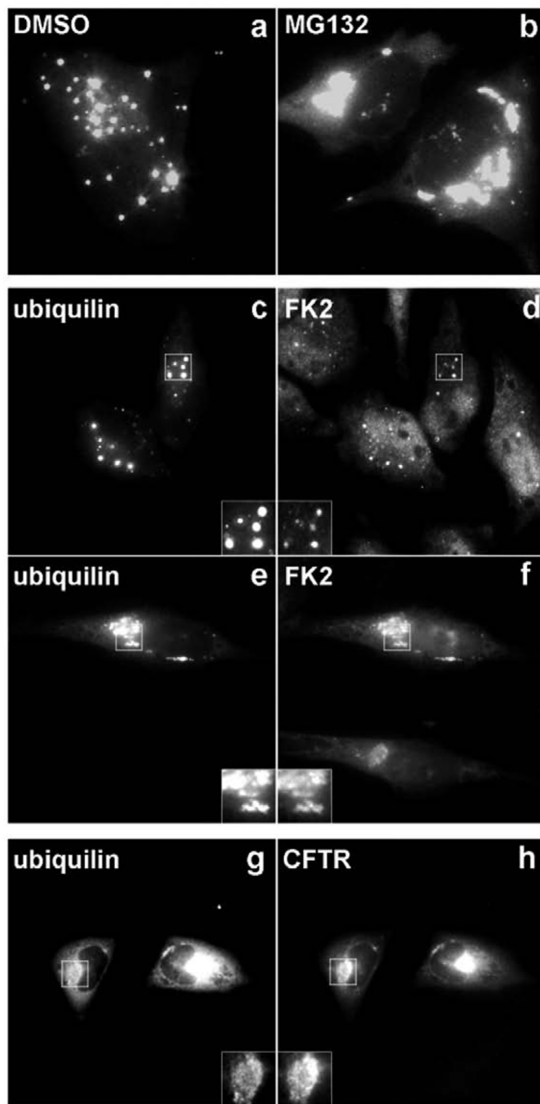

Fig. 8. GFP-ubiquilin is localized to aggresomes upon proteasome inhibition. HeLa cells transiently transfected with the GFP-ubiquilin construct (a-f) or with HA-ubiquilin and the GFP-CFTR constructs (g,h) were mock treated (0.1% DMSO) (a,c,d) or incubated overnight with 5 μM MG132 (b,e-h). Cells were fixed, permeabilized and processed for fluorescence microscopy using the FK2 anti-ubiquitin antibody (c-f) or the anti-HA antibody (g-h). The first antibodies were revealed by Cy3-labelled goat anti-mouse immunoglobulin secondary antibodies. (a-c,e,h) Green fluorescence emitted by GFP. (d,f,g) Red fluorescence emitted by Cy3.

Localization of Eps15 into ubiquilin-containing aggregates is UIM dependent

Our finding showing that the UIMs of Eps15 are required for the interaction with ubiquilin prompted us to examine whether the recruitment of Eps15 into the ubiquilin-containing cytoplasmic aggregates might be UIM dependent. We therefore examined the colocalization of GFP-ubiquilin with wild-type FLAG-Eps15 or with a FLAG-Eps15 deletion mutant lacking both UIM1 and UIM2 (FLAG-Eps15-ΔUIM1+2). As previously shown for endogenous Eps15 (Fig. 7a,b), the wild-type FLAG-Eps15 construct colocalized with GFP-ubiquilin to cytoplasmic aggregates (Fig. 10a,b). Strikingly, the FLAG-Eps15-ΔUIM1+2 mutant did not colocalized with GFP-ubiquilin to these structures (Fig. 10c,d). This result indicates that the colocalization of Eps15 with ubiquilin to cytoplasmic aggregates is UIM dependent.

Discussion

The UIM was first identified by bioinformatics analysis as a putative ubiquitin-binding motif based on its similarity to the ubiquitin-binding motif of the S5a proteasomal subunit. The UIM, either alone or in tandem, was found in endocytic proteins including epsins, Hrs, STAM and STAM2/Hbp, and their respective yeast counterparts Ent1/2, Vps27 and Hse1, as well as in Eps15 and Eps15R, whose yeast homologue Ede1p does not contain a UIM but instead possesses a ubiquitin-associated (UBA) domain (Hofmann and Falquet, 2001). Subsequently, the ability of these UIMs to bind recombinant ubiquitin and ubiquitinated proteins from cellular lysates was experimentally confirmed (Fig. 4) (Bilodeau et al., 2002; Katz et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Although Eps15 was shown to coimmunoprecipitate with ubiquitinated EGFR in a UIM-dependent manner (Sigismund et al., 2005), no direct binding of the UIMs of Eps15 to a ubiquitin moiety on the activated EGFR has been demonstrated. Although there is experimental evidence for the ability of the UIM of Hrs to interact with ubiquitinated cargoes in the limiting membrane of the early endosomes (Raiborg et al., 2002; Urbé et al., 2003), no specific binding partner of this UIM has been identified. Ubiquilin is therefore the first identified binding partner of the UIMs of Eps15, Hrs and Hbp. Moreover, we provide the first evidence that the UIMs of Eps15 and Hrs can bind a UBL domain.

Surprisingly, not all UIM-containing endocytic proteins seem to interact with the UBL domain of ubiquilin. Indeed, the UIM-containing protein epsin was not precipitated by GST-ubiquilin and nor was it colocalized with GFP-ubiquilin. Interestingly, epsin is localized to CCPs at the plasma membrane (Chen et al., 1998; Wendland, 2002) but it is not found on the limiting membrane of early endosomes, whereas the three UIM-containing proteins Hrs, Eps15 and Hbp have been found on the endosomal membrane (Raiborg et al., 2001), where they are part of a ternary complex (Bache et al., 2003). The association of ubiquilin with these three endocytic proteins and not with epsin might reflect a specific function addressed by ubiquilin in the recruitment (or sequestration) of these endocytic proteins. One possible explanation for the different binding of ubiquilin to different UIM-containing proteins is that there are subclasses of UIM with distinct conformations, which could preferentially bind ubiquitin rather than ubiquitin-

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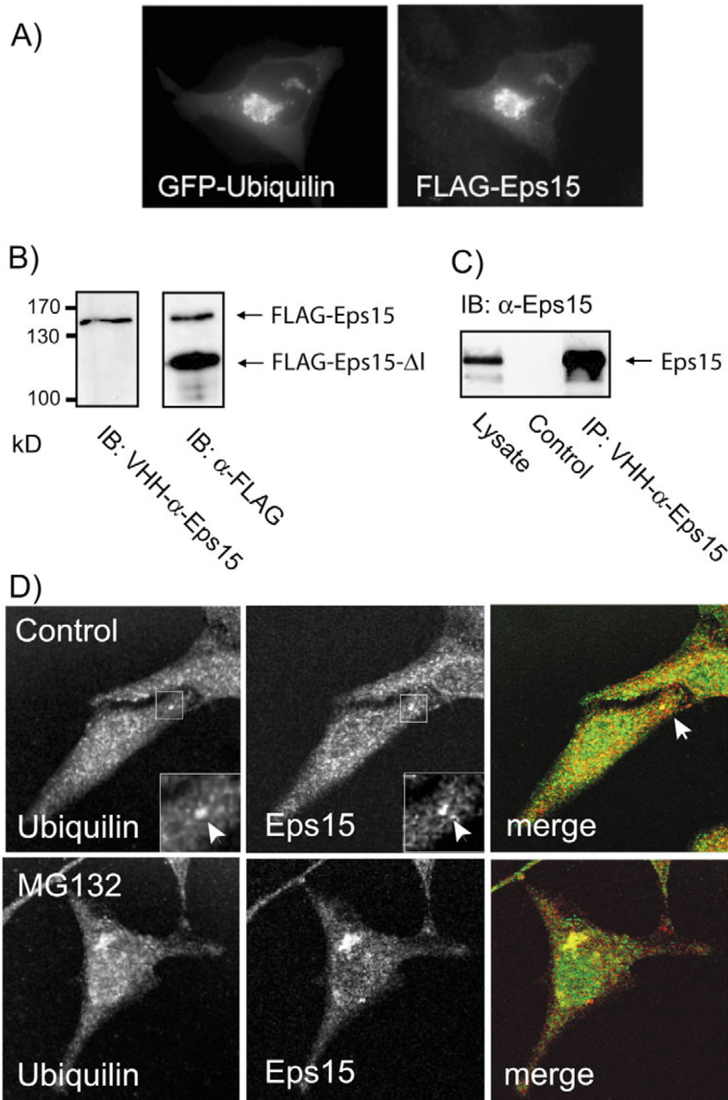

Fig. 9. Colocalization of endogenous Eps15 and endogenous ubiquilin to aggresomes. (A) GFP-ubiquilin and FLAG-Eps15 colocalize to aggresomes. HeLa cells transiently co-transfected with constructs encoding GFP-ubiquilin and the FLAG-Eps15 were incubated overnight with 5 μM MG132 and processed for fluorescence microscopy using the anti-FLAG antibody followed by a Cy3-labelled goat anti-mouse immunoglobulin secondary antibody. (left) Green fluorescence emitted by GFP. (right) Red fluorescence emitted by Cy3. (B) Characterization of the anti-Eps15 nanobody by western blot. Cos-1 cells were transiently co-transfected with wild-type FLAG-Eps15 and with FLAG-Eps15-ΔI (lacking the EH 1-3 domains). Total cellular lysates were subjected to SDS-PAGE and blotted onto PVDF membrane. (left) Immunodetection with the anti-Eps15 nanobody (IB: VHH-α-Eps15) that was detected using the anti-Myc antibody. (right) Immunodetection with the anti-FLAG antibody (α-FLAG). Notice that the llama VHH against Eps15 that was raised against domain I (EH1-3) of Eps15 does not recognize FLAG-Esp15-ΔI as expected. (C) Characterization of the anti-Eps15 nanobody by immunoprecipitation. Biotinylated anti-Eps15 nanobody was added to HeLa cellular lysates and precipitated using streptavidin-Sepharose beads (IP: VHH-α-Eps15). As a control, HeLa cellular lysates were precipitated with streptavidin-Sepharose beads in the absence of antibody (Control). The beads were subjected to SDS-PAGE and immunoblotting was performed with a rabbit anti-Eps15 antibody (IB: α-Eps15). Cellular lysates from HeLa were also loaded on the SDS-PAGE (Lysate). (D) HeLa cells were mocked treated (0.1% DMSO) (Control, top) or incubated overnight with 5 μM MG132 (bottom) and fixed with ice-cold methanol. Endogenous ubiquilin was detected using a rabbit anti-ubiquilin antibody revealed with Alexa-488-labelled goat anti-rabbit immunoglobulins secondary antibody (left). Endogenous Eps15 was detected using the VHH against Eps15, followed by mouse anti-Myc antibody revealed with Alexa-555-labelled goat anti-mouse immunoglobulin secondary antibody (centre). (right) Merged pictures, with yellow indicating colocalization of Eps15 and ubiquilin. The arrow indicates an aggregate in which Eps15 and ubiquilin colocalize.

like proteins. In line with this hypothesis, the first UIM of Eps15 does not bind to ubiquitinated proteins (Fig. 4B) (Polo et al., 2002) but does bind to the UBL of ubiquilin (Fig. 5C). By contrast, the second UIM of Eps15 preferentially binds ubiquitinated proteins rather than ubiquilin or the UBL (Fig. 4B, Fig. 5C). The difference in binding specificity of the two UIMs of Eps15 can be explained by their different sequence. Indeed, the UIM1 lacks the well-conserved central LXLAI/LXL motif present in the UIM2 and in many other UIM proteins (Hofmann and Falquet, 2001). Leucine residues surrounding this motif were previously shown to be involved

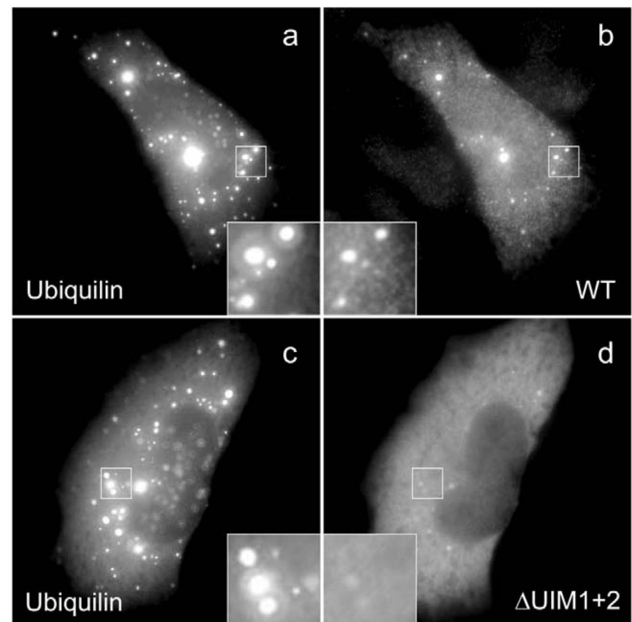

Fig. 10. UIM-dependent localization of Eps15 into ubiquilin-containing cytoplasmic aggregates. HeLa cells transiently co-transfected with the GFP-ubiquilin construct and with the FLAG-Eps15 (a,b) or the FLAG-Eps15-ΔUIM1+2 (c,d) construct were fixed, permeabilized and processed for fluorescence microscopy using the anti-FLAG antibody followed by Cy3-labelled goat anti-mouse immunoglobulin secondary antibodies. (a,c) Green fluorescence emitted by GFP. (b,d) Red fluorescence emitted by Cy3. Insets show higher magnifications of representative areas.

in ubiquitin binding (Shekhtman and Cowburn, 2002). It is possible that leucine-rich UIMs bind preferentially to ubiquitin, whereas other UIMs might bind better to ubiquitin-like domains.

Two families of ubiquitin-like proteins have been described. The type 1 ubiquitin-like proteins, such as SUMO-1 and Nedd8, are small proteins that share with ubiquitin the ability to be covalently attached to target proteins through their C-terminal glycine. The type-2 ubiquitin-like proteins, such as ubiquilin, possess a domain homologous to ubiquitin as part of their open reading frame. This UBL is often found at or close to the N-terminus of these proteins (Buchberger, 2002). Previous reports have shown that ubiquitin-like domains can bind to the UIM. The UBL domain of the type-2 ubiquitin-like proteins hHR23A and hHR23B, the human homologues of Rad23, was shown to bind the second UIM of the S5a proteasomal subunit (Fujiwara et al., 2004; Mueller and Feigon, 2003). The UBL domain of hPLIC2 (also known as ubiquilin 2) has also been shown to bind to S5a (Walters et al., 2002), but the involvement of the UIM1 or UIM2 of S5a in this interaction has not been investigated. In this report, we demonstrate that the UBL domain of ubiquilin can interact with the UIMs of Eps15 (Fig. 5C) and that its association to Hrs is UIM dependent (Fig. 6D). Furthermore, we demonstrate that the UIM-UBL interaction is direct, because recombinant His_6-Myc-UBL and His_6-Myc-ubiquilin were precipitated by GST-UIM constructs from Eps15 (Fig. 5C) and by a GST-Hrs-ΔCT construct but not by a GST-Hrs-ΔCT-ΔUIM construct (data not shown).

Our initial observation that the EH domains of Eps15 interact with ubiquilin in a yeast-two hybrid assay suggests that the interaction between Eps15 and ubiquilin might involve the first domain of Eps15. However, the EH domains of Eps15 are not essential for the interaction because their deletion does not prevent ubiquilin from interacting with Eps15. To our surprise, the deletion of the coiled-coil domain of Eps15 (domain II) disrupted the interaction with ubiquilin. The coiled-coil domain of Eps15 is responsible for Eps15 oligomerization (Tebar et al., 1997) and Eps15 is mainly found in dimers and tetramers (Cupers et al., 1997; Tebar et al., 1997). It is possible that dimerization of Eps15 is necessary to increase the avidity of its UIM for the UBL domain of ubiquilin. Indeed, oligomers of Eps15 could present multiple copies of the UIM to the UBL. In that respect, it is interesting that the first crystal structure of a UIM (the second UIM of Vps27) revealed a tetrameric structure (Fisher et al., 2003). In the case of Eps15, dimerization or tetramerization might promote the formation of a UIM tetramer. Although Hrs and Hbp have only one UIM, the fact that they associate with each other and with Eps15 might also promote the oligomerization of UIMs. The idea that multiple copies of UIMs are required for interaction with UBL or with multiple ubiquitins is reinforced by the observation that individual UIMs have relatively low affinity for monoubiquitin (Fisher et al., 2003; Polo et al., 2002; Raiborg et al., 2002). In this work, we show that the mutation of either UIM1 or UIM2 in the context of full-length Eps15 disrupts the interaction with ubiquilin (Fig. 4A) even though each UIM is sufficient to interact with ubiquilin, as shown in GST pull-down assays (Fig. 4B, Fig. 5C). This suggests that a tandem repeat of UIMs is required for a stable interaction with the UBL domain of ubiquilin.

The capacity of Eps15 to be recruited to ubiquitin-rich cytoplasmic aggregates has been previously reported. An engineered aggregation-prone protein consisting of an extended polyglutamine track of ataxin-3 fused to cyan fluorescent protein (CFP-Q78) formed aggregates that stained positive for ubiquitin and that recruited Eps15 (Donaldson et al., 2003). Furthermore, UIM mutants of ataxin-3 were not recruited into ubiquitin-rich aggregates (Donaldson et al., 2003). This example is consistent with our observations that endogenous Eps15 is recruited to ubiquilin-containing cytoplasmic aggregates that contain ubiquitinated proteins (Figs 7, 8) and that deletion of both UIMs abolishes the recruitment of Eps15 into these aggregates (Fig. 10). Moreover, endogenous ubiquilin and Eps15 did colocalize to cytoplasmic aggregates and both proteins were found in aggresomes upon proteasome inhibition (Fig. 9), arguing that our observations with GFP-ubiquilin are physiologically relevant. Altogether, our data suggest that the recruitment of UIM-containing endocytic proteins into ubiquilin-containing cytoplasmic aggregates represents the physiological capacity of these proteins to interact with ubiquitin-like proteins via their UIMs.

Ubiquilin has been detected in Lewy bodies of brains affected with Parkinson's disease and with diffuse Lewy bodies disease (Mah et al., 2000). Consistent with our data, it has been shown that endogenous ubiquilin forms cytoplasmic aggregates in HeLa cells (Mah et al., 2000) and that overexpressed ubiquilin localizes to ubiquitin-positive structures and is present in PS2-containing aggresomes (Massey et al., 2004). Many neurodegenerative diseases are characterized by the accumulation of ubiquitin-rich protein aggregates in affected neurons, resulting in neurodegeneration and subsequent neuronal cell death. It is conceivable that the sequestration of UIM-containing endocytic proteins into these aggregates divert them from their cellular site of action, resulting in an altered endosome-lysosome pathway. In line with this idea, it was recently shown that aggregation of expanded polyglutamine polypeptides led to defects in endocytosis (Meriin et al., 2003). The question of whether aggregation of ubiquilin and subsequent sequestration of UIM-containing proteins into these aggregates represents a mechanism involved in the pathogenesis of neurodegenerative disease is intriguing and awaits further investigation.

Alternatively, the presence of UIM-containing proteins in cytoplasmic aggregates might reflect a role for these endocytic proteins in the clearance of these aggregates. It has long been suggested that cytoplasmic aggregates and aggresomes are cleared from the cell by the autophagic route (Garcia-Mata et al., 2002; Kopito, 2000). Recently, experimental evidence has clearly established a role for autophagy in the clearance of polyglutamine aggregates (Ravikumar et al., 2004) and of aggresomes from Schwann cells and fibroblasts (Fortun et al., 2003). Autophagy is the major process by which cytoplasmic components, including organelles, are degraded. Cytoplasmic cargos are engulfed by membranes to form autophagosomes that subsequently fuse with lysosomes, where degradation occurs. It is likely that the autophagic and endocytic pathways converge before the lysosomal level, because fusion of autophagic vesicles with endosomes has been shown by immunoelectron microscopy (Liou et al., 1997). Given the roles of Hrs, Hbp and Eps15 in the endocytic pathway, it is tempting to speculate that their presence in ubiquitin-rich

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cytoplasmic aggregates or in aggresomes might reflect a role either in the recruitment of membranes to the cytoplasmic aggregates and/or in the fusion of autophagic vesicles with the endosomal/lysosomal compartments. Finally, it is of interest that ubiquilin is a binding partner of the serine/threonine kinase mTOR, which is involved in the induction of autophagy (Wu et al., 2002).

In conclusion, we have identified ubiquilin as a common binding partner of a subfamily of UIM-containing endocytic proteins. The identification of an interaction between the UIM of endocytic proteins and the UBL domain extends the concept of a 'ubiquitin network'. This network of proteins was originally based upon interactions between ubiquitin and ubiquitin-binding regions such as the UIM (Polo et al., 2003). The UIM-UBL interaction expands this network to an 'ubiquitin-like network', where ubiquitin-like proteins and ubiquitin-binding regions are likely to play a role in the regulation of biological processes such as intracellular trafficking.

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