EMBO reports

scientific report

The UBL domain of PLIC-1 regulates aggresome formation

Renu Heir[1], Celine Ablasou[2], Emilie Dumontier[1], Meghan Elliott[1], Christine Fagotto-Kaufmann[1] & Fiona K. Bedford[1+]

[1]Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada, and [2]Laboratoire d'Immunologie Equipe d'Accueil 2686, Faculte de Medecine, Pole Recherche, Lille, France

Defects in protein folding and the proteasomal pathway have been linked with many neurodegenerative diseases. PLIC-1 (protein linking IAP to the cytoskeleton) is a ubiquitin-like protein that binds to the ubiquitin-interacting motif (UIM) of the proteasomal subunit S5a. Here, we show that PLIC-1 also binds to the UIM proteins ataxin 3—a deubiquitinating enzyme—HSJ1a—a co-chaperone—and EPS15 (epidermal growth factor substrate 15)—an endocytic protein. Using a polyglutamine (polyQ) disease model, we found that both endogenous PLIC-1 and EPS15 localize to perinuclear aggresomes, and that polyQ enhances their *in vivo* interaction. We show that knockdown of PLIC-1 and EPS15 by RNA interference reduces aggresome formation. In addition, PLIC-1$^{\Delta UBL}$ functions as a dominant-negative mutant, blocking both polyQ transport to aggresomes and the association of EPS15 with dispersed aggregates. We also show that PLIC-1 is upregulated by arsenite-induced protein misfolding. These results indicate a role for PLIC-1 in the protein aggregation-stress pathway, and we propose a novel function for the ubiquitin-like (UBL) domain—by means of UBL–UIM interactions—in transport to aggresomes.

Keywords: PLIC-1; EPS15; aggresomes

EMBO reports (2006) **7**, 1252–1258. doi:10.1038/sj.embor.7400823

INTRODUCTION

Ubiquitin modification of proteins is both rapid and reversible, and a regulatory role for ubiquitination is akin to phosphorylation. Ubiquitination can signal proteins for degradation (Pickart, 2001), and regulate protein interactions and intracellular localization (Di Fiore *et al*, 2003). A domain homologous to ubiquitin is also found in proteins of the type II ubiquitin-like (UBL) family (Hartmann-Petersen & Gordon, 2004). Sequence and structural homology indicates that UBL domains mimic aspects of ubiquitin function, in particular its interaction with the proteasome (Walters *et al*, 2002). Included in this UBL family is PLIC-1 (protein linking IAP to the cytoskeleton (Wu *et al*, 1999; Bedford *et al*, 2001), also known as ubiquilin-1 (Mah *et al*, 2000). Structurally, PLIC-1 encodes an amino-terminal UBL domain that binds to the second ubiquitin-interacting (UIM-2) motif of S5a, a proteasomal 19S cap subunit (Walters *et al*, 2002). PLIC-1 also encodes a region rich in asparagine–proline repeats and a carboxy ubiquitin-associated (UBA) domain, which binds to polyubiquitinated proteins (Chen *et al*, 2001). The ability of PLIC-1 to bind to both ubiquitinated proteins and the proteasome has suggested a role for PLIC-1 as a proteasomal shuttling factor.

Several observations indicate that the UBL domain is crucial for the function of PLIC-1. The yeast homologue of PLIC-1—Dsk2p—was identified through a function-disrupting mutation in the UBL domain (Biggins *et al*, 1996). The UBL domain is also highly conserved between PLIC-1 and its homologues in sequence (Kleijnen *et al*, 2000), structure and proteasomal binding (Walters *et al*, 2002). In addition, an interesting observation is that the UIM domain of S5a recognizes both the UBL domain of PLIC (Walters *et al*, 2002) and ubiquitin (Fisher *et al*, 2003) through a conserved surface.

UIM domains have been identified in various proteins and their ubiquitin-binding abilities confirmed (Hofmann & Falquet, 2001). These include the epidermal growth factor substrate 15 (EPS15; Polo *et al*, 2002), the deubiquitinating enzyme ataxin 3 (AT3; Burnett & Pittman, 2005) and the co-chaperone human neuron-specific DNAJ-like protein 1a (HSJ1a; Westhoff *et al*, 2005). The identification of UIMs in proteins other than S5a raises the possibility that there might be many UIM-containing proteins that bind to the UBL domain of PLIC-1.

Here, we report that the UBL domain of PLIC-1 binds to the UIM domains of EPS15, AT3 and HSJ1a. Using a polyglutamine (polyQ) disease-model protein, we examined the role of PLIC-1 and the endocytic UIM protein EPS15 in aggresome formation, and identified a novel function for the UBL domain of PLIC-1 in aggregate transport.

[1]Department of Anatomy and Cell Biology, McGill University, 3640 University Street, Montreal, Quebec H3A2B2, Canada
[2]Laboratoire d'Immunologie Equipe d'Accueil 2686, Faculte de Medecine, Pole Recherche, 1 Place de Verdun, 59045 Lille, France
[+]Corresponding author. Tel: +1 514 398 1426; Fax: +1 514 398 5047;
E-mail: fiona.bedford@mcgill.ca

Received 4 May 2005; revised 4 September 2006; accepted 4 September 2006; published online 3 November 2006

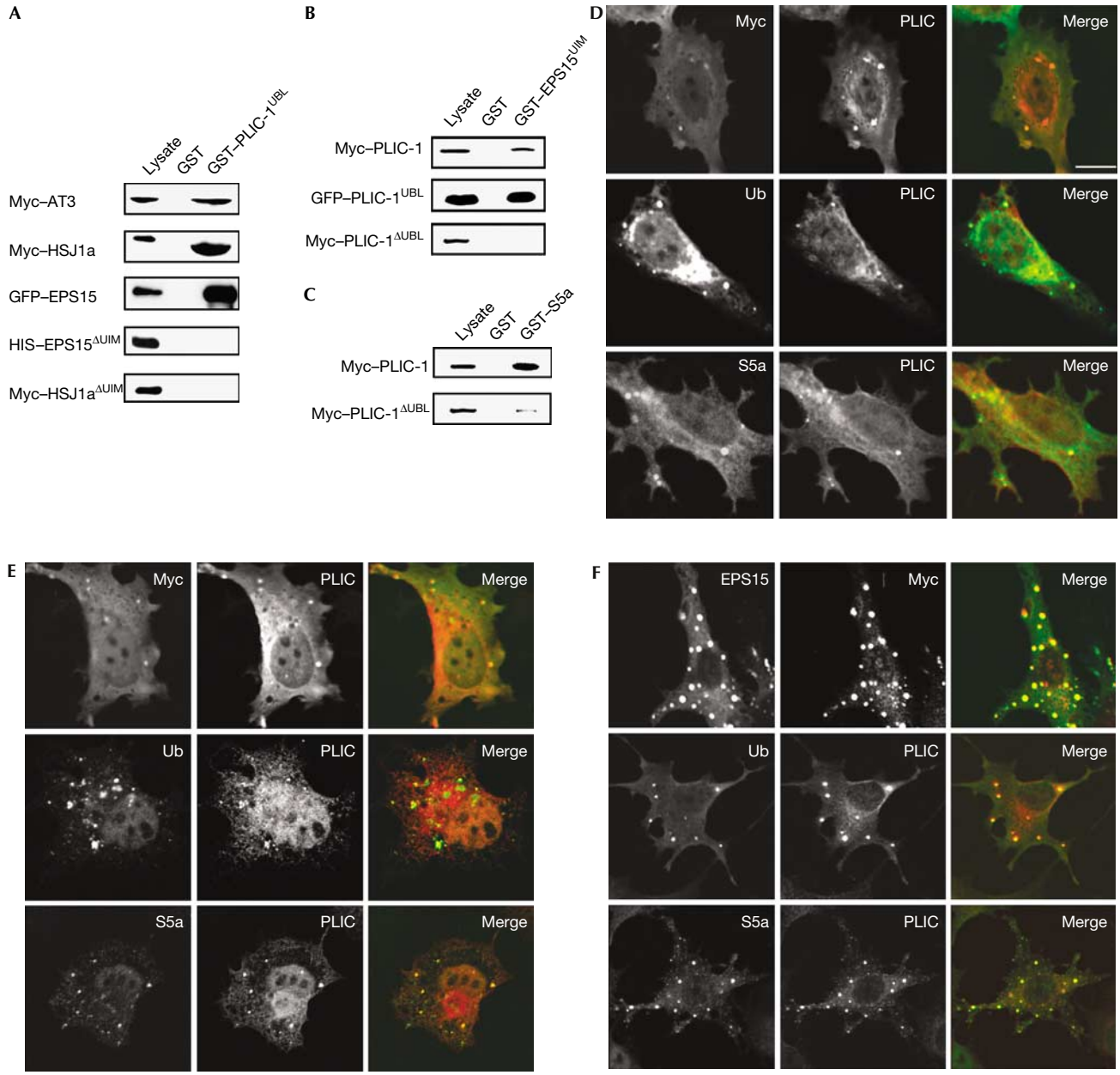

Fig 1 | The ubiquitin-like domain of PLIC-1 binds to multiple proteins containing the ubiquitin-interacting motif. Lysates from baby hamster kidney cells transfected with the indicated proteins were pulled down with GST fusion proteins, followed by western blotting with antibodies to the designated epitope tags. (**A**) *In vitro* binding of Myc–AT3, Myc–HSJ1a, GFP–EPS15, His–EPS15$^{\Delta UIM}$ and Myc–HSJ1a$^{\Delta UIM}$ to immobilized GST–PLIC-1^{UBL} and GST. (**B**) *In vitro* binding of Myc–PLIC-1, GFP–PLIC-1^{UBL} and Myc–PLIC-1$^{\Delta UBL}$ to immobilized GST–EPS15^{UIM} and GST. (**C**) *In vitro* binding of Myc–PLIC-1 and Myc–PLIC-1$^{\Delta UBL}$ to GST–S5a and GST. Lysate is 5% of input for GFP–EPS15 (**A**) and 10% for all others. PLIC-1–UIM protein structures resemble aggresomes. BHK cells transfected with (**D**) PLIC-1 and Myc–AT3, (**E**) PLIC-1 and Myc–HSJ1a and (**F**) PLIC-1 and His–EPS15 were stained for colocalization, ubiquitinated proteins (Ub) and the proteasomal subunit S5a as designated on the panels. Scale bar, 10 μm. AT3, ataxin 3; BHK cells, baby hamster kidney cells; EPS15, epidermal growth factor substrate 15; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HIS, 6× histidine; HSJ1a, human neuron-specific DNAJ-like protein 1a; PLIC-1, protein linking IAP to the cytoskeleton; S5a, proteasomal subunit S5a; UBL, ubiquitin-like; UIM, ubiquitin-interacting motif.

RESULTS

The UBL domain binds to multiple UIM proteins

PLIC-1 has been shown to bind to the UIM domains of S5a (Walters *et al*, 2002). To test whether UIM protein binding is a general property of the UBL domain, we carried out glutathione *S*-transferase (GST) pulldowns using the UIM proteins AT3, HSJ1a and EPS15. GST fused to the UBL domain of PLIC-1 (GST–PLIC-1^{UBL}) pulled down Myc–AT3 and Myc–HSJ1a, and also green

fluorescent protein (GFP)–EPS15 (Fig 1A). Deletion of the UIM domains of either HSJ1a or EPS15 resulted in the loss of binding of these proteins to GST–PLIC-1^{UBL}. In addition, GST–EPS15^{UIM} pulled down Myc–PLIC-1 and GFP–PLIC-1^{UBL} but not a Myc–PLIC-1$^{\Delta UBL}$ mutant (Fig 1B). As a control, we used GST–S5a, which, as predicted (Kleijnen *et al*, 2003), bound to Myc–PLIC-1 and to Myc–PLIC-1$^{\Delta UBL}$, although with lower affinity (Fig 1C). These results indicate that binding of UIM proteins is a common feature of the UBL domain of PLIC-1.

PLIC-1–UIM protein structures resemble aggresomes

AT3 and HSJ1a are associated with the clearance of misfolded aggregation-prone proteins (Burnett & Pittman, 2005; Westhoff *et al*, 2005), and EPS15 is associated with the sorting of monoubiquitinated proteins for endocytosis and lysosomal degradation (Polo *et al*, 2002). Alone, PLIC-1 showed diffuse expression (supplementary Fig S1a online), whereas coexpression with AT3 or HSJ1a resulted in the formation of small punctate structures containing both proteins (Fig 1D,E). To examine whether the constituents of these punctate structures were associated with the clearance of misfolded aggregation-prone proteins (Johnston *et al*, 1998), as suggested by AT3 and HSJ1a functions, we co-stained with antibodies to ubiquitinated proteins and the proteasomal subunit S5a, and found that they stained positively for both (Fig 1D,E).

Coexpression of PLIC-1 and EPS15 also resulted in the localization of both proteins in punctate structures (Fig 1F), which were more pronounced than with AT3 and HSJ1a; EPS15 expression alone did not induce such a phenotype (supplementary Fig S1b online). Enlarged early endosomes are commonly associated with the overexpression of endosome-associated proteins. However, these structures did not stain for the early endosomal antigen-1 (supplementary Fig S2 online) but instead for ubiquitin and S5a (Fig 1F), similar to the HSJ1a and AT3 structures.

The EPS15 PLIC-1 structures varied in size; therefore, we postulated that they coalesced to form larger structures. To investigate this, we used live imaging of cells transfected with GFP–EPS15 and PLIC-1 (supplementary Fig S3 and Movie 1 online). We observed that small GFP-labelled structures formed and then seemed to be transported, merging into larger structures close to the nucleus. Aggresomes are characterized by the presence of ubiquitinated misfolded proteins and the proteasome, and are formed by the retrograde transport of small aggregates on microtubules to the microtubule-organizing centre (MTOC; Johnston *et al*, 1998). Nocodazole depolymerization of microtubules, which blocks aggresome formation, inhibited the merging of these structures (supplementary Fig S3 and Movie 2 online); therefore, these EPS15 PLIC-1 structures show several defining characteristics of aggresomes.

Endogenous PLIC-1 and EPS15 localize to aggresomes

AT3 and HSJ1a have been found at aggresomes formed either by an elongated tract of glutamines (polyQ) or by the ΔF508 cystic fibrosis transmembrane conductance regulator mutant and are involved in aggresome biogenesis (Burnett & Pittman, 2005; Westhoff *et al*, 2005). To investigate whether endogenous PLIC-1 and EPS15 are similarly associated with aggresomes, we used an AU1 epitope tagged expanded polyQ tract protein (AU1-Q78; Toulouse *et al*, 2005) to induce aggresome formation. AU1-Q78

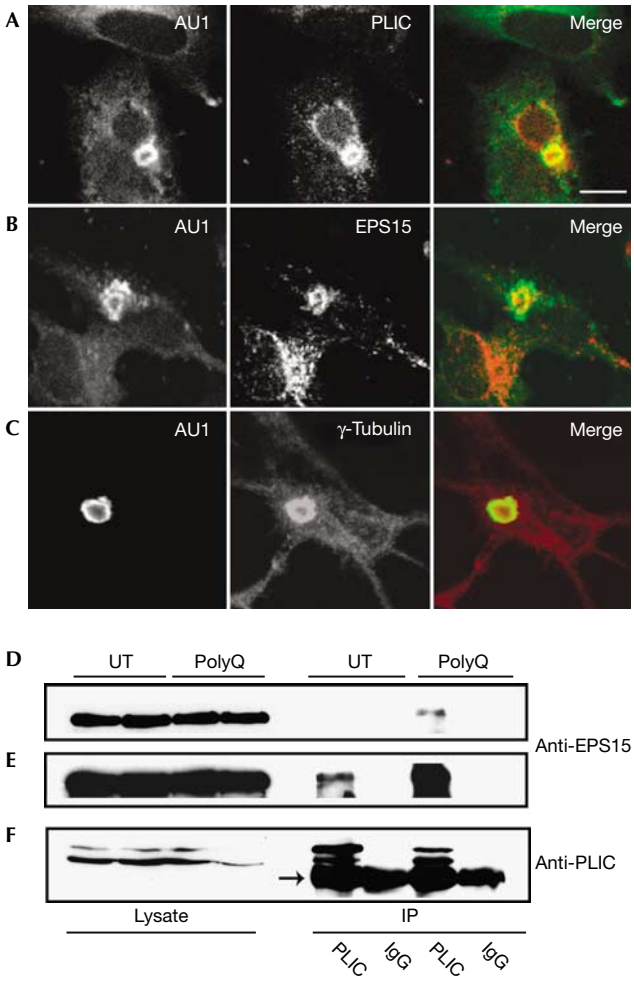

Fig 2 | Endogenous PLIC-1 and epidermal growth factor substrate 15 interact and localize to aggresomes. Baby hamster kidney cells were transfected with AU1-Q78 and stained for (**A**) AU1 and PLIC-1, (**B**) AU1 and EPS15 or (**C**) AU1 and γ-tubulin. All proteins localize to aggresomes. Scale bar, 10 μm. Aggregation modulates endogenous PLIC-1 and EPS15 interaction. Lysates from untransfected (UT) or AU1-Q78 (polyQ)-transfected BHK cells were immunoprecipitated (IP) with anti-PLIC-1 or control rabbit IgG and subjected to western blotting with antibodies to EPS15 (**D**,**E**) and PLIC-1 (**F**). (**D**) Representation of a longer exposure of (**E**); the arrow indicates heavy-chain IgG. AU1, epitope tag; EPS15, epidermal growth factor substrate 15; PLIC-1, protein linking IAP to the cytoskeleton.

formed single, large perinuclear aggresomes, which co-stained for both endogenous PLIC-1 and EPS15 (Fig 2A,B). These aggresomes were also characterized by staining with the MTOC marker γ-tubulin (Fig 2C), which is a marker of aggresomes (Johnston *et al*, 1998). These results show that endogenous PLIC-1 and EPS15 localize to γ-tubulin-positive aggresomes.

Aggregation modulates PLIC-1 and EPS15 interaction

As PLIC-1 and EPS15 interacted *in vitro* (Fig 1A), colocalized when overexpressed (Fig 1F), and the endogenous proteins localized to polyQ aggresomes (Fig 2A,B), we examined whether these two

proteins interacted endogenously by co-immunoprecipitation and whether aggregation-prone proteins modulate this interaction. We found that endogenous EPS15 co-immunoprecipitated with PLIC-1 and that more EPS15 was bound in AU1-Q78-expressing cells (Fig 2D–F). Neither endogenous EPS15 nor PLIC-1 was detected in control rabbit IgG immunoprecipitates, and AU1-Q78 expression was confirmed by western blot (data not shown). These results indicate that endogenous EPS15 and PLIC-1 interact and that their association is enhanced by the presence of an aggregation-prone protein.

PLIC-1 and EPS15 regulate aggresome formation

To investigate whether PLIC-1 and EPS15 are required for aggresome formation, we used RNA interference (RNAi) to knock down their expression, quantified aggresome formation and compared the results with control cells transfected with scrambled RNAi, which were assigned a value of 1. We found that knockdown of PLIC-1 (Fig 3A) significantly inhibited aggresome formation to 0.724 ± 0.070 of control ($P < 0.05$; Fig 3C). Knockdown of EPS15 (Fig 3B) also significantly inhibited aggresome formation to 0.530 ± 0.068 of control ($P < 0.01$; Fig 3C). No significant difference in aggresome formation was found between PLIC-1 and EPS15 knockdown conditions. These reductions were not an effect of cell death, but were due to an increase in the number of cells with diffuse AUI-Q78 expression (Fig 3D). These results indicate that PLIC-1 and EPS15 regulate aggresome formation.

The UBL domain mediates aggregate transport

To investigate whether the UBL domain—which mediates the EPS15 interaction—is required for aggresome formation, we used a PLIC-1$^{\Delta UBL}$ mutant that was compromised in its ability to bind to UIM motifs (Fig 1B), but not to ubiquitinated proteins (supplementary Fig S4 online). When coexpressed with AUI-Q78, PLIC-1$^{\Delta UBL}$ severely reduced aggresome formation: γ-tubulin-positive aggresomes formed in less than 1% of co-transfected cells. Instead, we observed numerous AUI-Q78- and Myc–PLIC-1$^{\Delta UBL}$-positive cytoplasmic aggregates (Fig 4A), which did not stain for γ-tubulin (data not shown). When coexpressed with AUI-Q78, Myc–PLIC-1 did not induce this phenotype (Fig 4B), with aggresomes forming in approximately 30% of co-transfected cells. These results indicate that PLIC-1 interacts with protein aggregates before MTOC transport and that the observed Q78-PLIC-1$^{\Delta UBL}$ phenotype is a result of aggregate sequestration arising from a block in transport. To confirm that endogenous PLIC-1 recognizes protein aggregates before their MTOC transit, we treated AUI-Q78-transfected cells with nocodazole and then stained for PLIC-1 (Fig 4C). Under these conditions, dispersed AU1-Q78 aggregates formed that co-stained for endogenous PLIC-1.

To examine whether the Q78-PLIC-1$^{\Delta UBL}$ dispersed aggregates resulted from a block in UBL–UIM interactions, we stained transfected cells for EPS15 and found that Q78-PLIC-1$^{\Delta UBL}$ aggregates were negative for endogenous EPS15 (Fig 4D), but positive for the proteasome (data not shown). By contrast, we found that EPS15 was normally localized to both AU1-Q78 aggresomes and dispersed aggregates (Fig 4E). These results indicate that PLIC-1$^{\Delta UBL}$ blocks both aggregate transport to the MTOC and EPS15 localization to aggregates. Therefore, PLIC-1$^{\Delta UBL}$ is a dominant-negative mutant of this process.

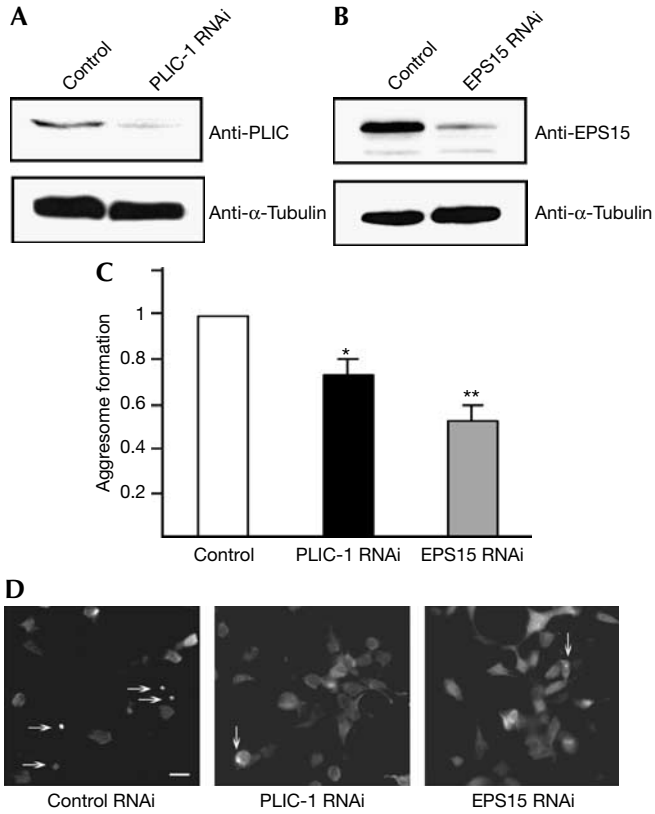

Fig 3 | PLIC-1 and epidermal growth factor substrate 15 regulate aggresome formation. Lysates from human embryonic kidney 293 cells transfected with AUI-Q78 and (**A**) control or PLIC-1 RNAi duplexes or (**B**) control or EPS15 RNAi duplexes were western blotted with (**A**) PLIC-1 and (**B**) EPS15 antibodies. α-Tubulin (**A**,**B**) was used as a loading control. (**C**) Cells transfected as in (**A**,**B**) were immunostained with AUI antibodies and 20 random fields of view were quantified for the number of cells containing AUI-Q78 aggresomes. RNAi-transfected cells were normalized to control (arbitrarily assigned a value of 1) for each experiment ± s.e.m. ($n = 3$ or more), between-subject analysis of variance was carried out and significant differences from control were found (*$P < 0.05$, **$P < 0.01$) using the Tukey's honestly significant difference *post hoc* test. (**D**) Images of cells transfected as in (**A**,**B**) and immunostained with AUI antibodies. Fewer cells form aggresomes (arrows) on PLIC-1 and EPS15 knockdown. Also note the increase in cells with diffuse AUI-Q78 expression in these panels. Scale bar, 30 μm. EPS15, epidermal growth factor substrate 15; PLIC-1, protein linking IAP to the cytoskeleton; RNAi, RNA interference.

PLIC-1 responds to general misfolded protein stress

Our observations have indicated that PLIC-1 recognizes aggregated proteins. This led us to investigate whether PLIC-1 expression is regulated by general cytoplasmic misfolded protein stress, which can be induced by using the heavy-metal poison arsenite (Novoa *et al*, 2003). To test this, we carried out semiquantitative reverse transcription–PCR (RT–PCR) on RNA isolated from untreated and arsenite-treated cells (Fig 5A); we found that PLIC-1 expression was increased 2.3 ± 0.3-fold by arsenite treatment (Fig 5B). As a positive control, we followed the

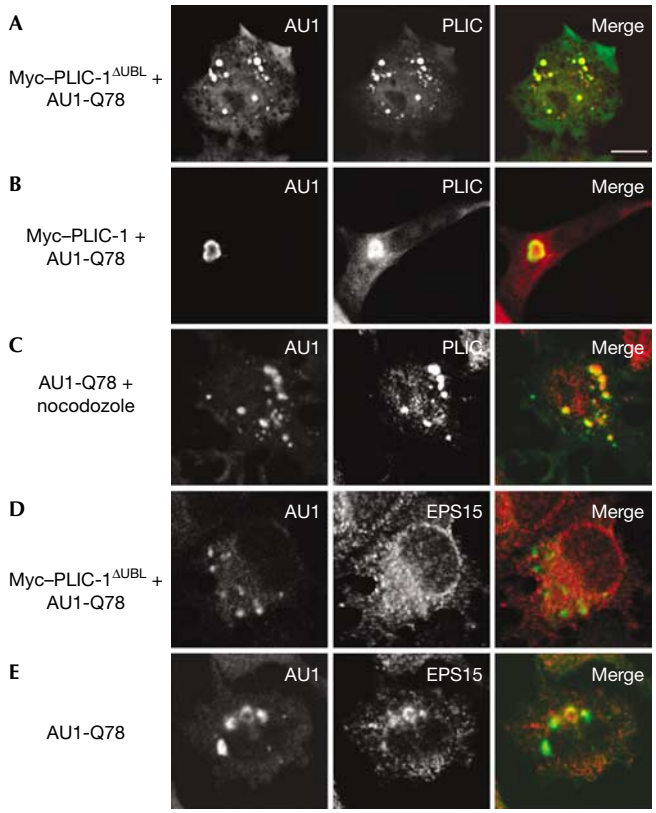

Fig 4 | The ubiquitin-like domain of PLIC-1 mediates aggregate transport. (**A**) Baby hamster kidney cells transfected with Myc–PLIC-1$^{\Delta UBL}$ and AUI-Q78 were stained for AU1 and PLIC-1. (**B**) Myc–PLIC-1- and AUI-Q78-transfected cells were stained for AU1 and PLIC-1. (**C**) AU1-Q78-transfected cells were treated with nocodazole (1 μM, 2 h) and stained for AU1 and PLIC-1. (**D**) Myc–PLIC-1$^{\Delta UBL}$- and AUI-Q78-transfected cells were stained for AU1 and EPS15. (**E**) AU1-Q78-transfected cells were stained for AU1 and EPS15. Scale bar, 10 μm. AU1, epitope tag; EPS15, epidermal growth factor substrate 15; PLIC-1, protein linking IAP to the cytoskeleton; UBL, ubiquitin-like.

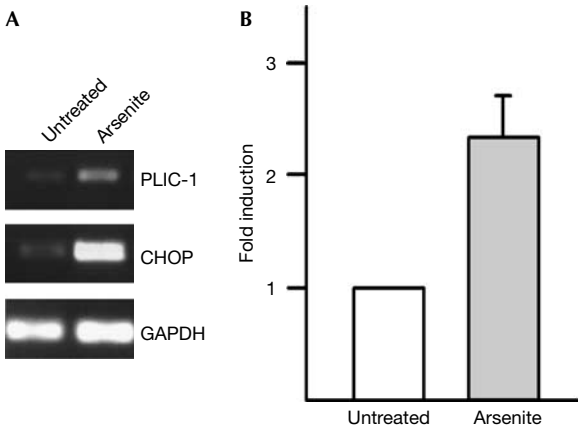

Fig 5 | PLIC-1 responds to general misfolded protein stress. (**A**) Semiquantitative reverse transcription–PCR of RNA from untreated or arsenite-treated (50 μM, 14 h) human hepatoma cells (HepG2) for PLIC-1, CHOP and GAPDH. (**B**) Fold induction of PLIC-1 (mean ± s.e.m., $n = 3$) by arsenite treatment normalized to GAPDH levels. CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLIC-1, protein linking IAP to the cytoskeleton.

induction of the C/EBP homologous protein (CHOP), which was potently upregulated by arsenite (Novoa *et al*, 2003); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. These results show that PLIC-1 expression is responsive to the arsenite-induced accumulation of misfolded proteins.

DISCUSSION

Protein aggregation is a defining characteristic of most neurodegenerative disorders (Ross & Poirier, 2004), and PLIC-1 is genetically associated with one such disease—Alzheimer's disease (Bertram *et al*, 2005); therefore, our findings are significant to understanding neurodegenerative disease progression. We show that (i) PLIC-1 expression is upregulated in response to an inducer of the general misfolded protein response pathway, (ii) the UBL domain of PLIC-1 binds to UIMs of several proteins and forms complexes *in vivo* that are sensitive to protein aggregation with the endocytic protein EPS15, (iii) endogenous PLIC-1 and EPS15 are recruited to MTOC-localized polyQ aggresomes, (iv) knockdown of PLIC-1 or EPS15 by RNAi affects aggresome formation, and (v) the loss of the UBL domain of PLIC-1 has a dominant-negative effect on aggregate transport and localization of EPS15 to aggregates. Together, these results indicate that PLIC-1 is a component of the misfolded protein response and that UBL-dependent interactions with EPS15 are important for its function in this pathway.

Identifying this novel function of the UBL domain of PLIC-1 and its binding to UIM proteins other than S5a (Fig 1) suggests that UIM interactions might be a broad property of the UBL domain. Therefore, it will be interesting to examine whether this UBL interacts with other ubiquitin-related domains and also the nature of these interactions. Although an interaction between recombinant EPS15 and PLIC-1 has been reported previously (Regan-Klapisz *et al*, 2005), here we show that the endogenous proteins interact and that this interaction is enhanced in response to an aggregation-prone protein (Fig 2D–F). The finding that this component of the endocytic machinery can modulate aggresome formation is interesting in the light of a previous report, which demonstrated that the loss of the yeast homologue of EPS15 enhances protein aggregate toxicity (Meriin *et al*, 2003). Future investigation is needed to examine whether other endocytic components might be required for this role of EPS15.

The observed effects of PLIC-1$^{\Delta UBL}$ on aggresome formation (Fig 4) suggest that PLIC-1 sequesters aggregated proteins, when compromised for its UBL-dependent interactions. Simple loss of S5a binding to PLIC-1$^{\Delta UBL}$, leading to impaired proteasomal degradation of AU1-Q78, cannot be reconciled with these results, as pharmacological inhibition of the proteasome promotes aggresome formation (Johnston *et al*, 1998). Furthermore, the proteasome still binds to PLIC-1$^{\Delta UBL}$ (Fig 1C) and is localized to PLIC-1$^{\Delta UBL}$ aggregates (data not shown). The link between PLIC-1 and aggregate transport is not unexpected given its ability to recognize ubiquitinated proteins and that misfolded proteins are generally ubiquitinated before degradation

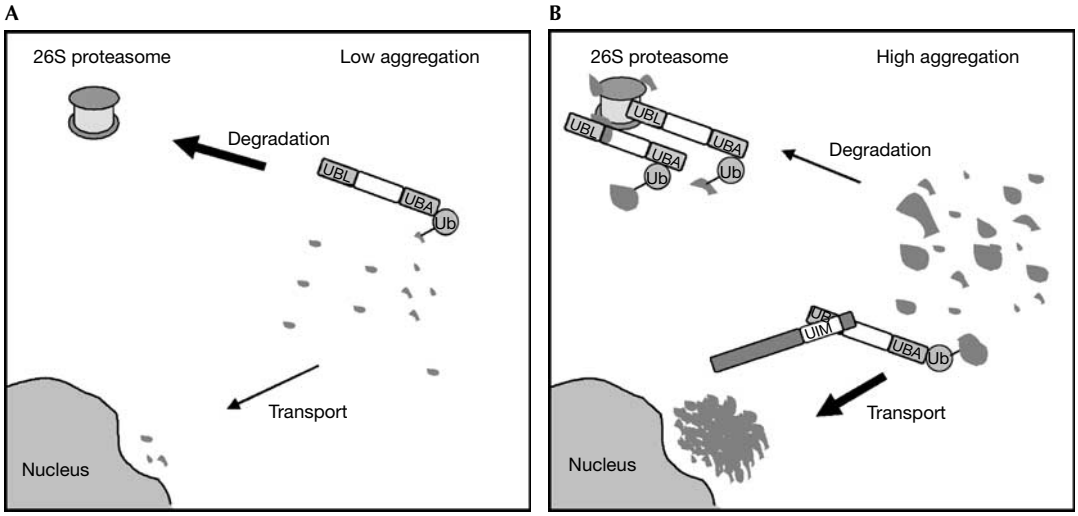

Fig 6 | Model for the role of PLIC-1 aggresome formation. (**A**) Under low levels of protein aggregation, the UBL domain of PLIC-1 shuttles ubiquitinated misfolded proteins to the proteasome through the UIM of S5a. (**B**) Under high levels, larger ubiquitinated aggregates overwhelm the proteasome, leading to the UBL domain being available for binding to other UIM proteins, which promote aggregate transport to the perinuclear aggresome. PLIC-1, protein linking IAP to the cytoskeleton; Ub, ubiquitin; UBA, ubiquitin-associated domain; UBL, ubiquitin-like; UIM, ubiquitin-interacting motif.

(Layfield *et al*, 2005). However, it remains to be determined precisely how AU1-Q78 aggregates transport to the MTOC and how aggresome formation is blocked.

Altogether, our findings support a model (Fig 6) that proposes a dual function for the UBL domain of PLIC-1 in both proteasomal turnover and transport to the aggresome by competitive UBL–UIM interactions. Under low levels of protein aggregation, PLIC-1 promotes the shuttling of aggregates to the proteasome for their degradation through S5a binding. By contrast, under high levels of protein aggregation, the proteasome becomes overwhelmed and alternative interactions of the UBL domain of PLIC-1, such as with EPS15, are enhanced, promoting the deposition of protein aggregates at the aggresome. Recent data suggest that efficient autophagic removal of the aggresome would follow, reducing cellular toxicity (Iwata *et al*, 2005).

In summary, accumulating evidence (Bertram *et al*, 2005; Wang *et al*, 2006), including the findings presented in this report, indicate that PLIC-1 is an important component of the quality control machinery that controls protein aggregation, and prevents cell death and disease.

METHODS

Cell culture and treatments. Baby hamster kidney (BHK) cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 5% CO_2 supplemented with 5% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine, whereas human embryonic kidney 293 (HEK293) cells and human hepatoma (HepG2) cells were cultured in DMEM. Transfections were carried out with Lipofectamine 2000 (Invitrogen). To induce misfolded protein stress, HepG2 cells were treated with 50 μM arsenite or DMSO for 14 h. For microtubule destabilization, cells were incubated with nocodazole (1 μM) for 2 h.

Antibodies and plasmids. For details, see the supplementary information online.

Immunofluorescence. BHK cells plated on poly-L-lysine-coated coverslips were processed for immunofluorescence as described by Bedford *et al* (2001), except for γ-tubulin staining, for which cells were fixed in 1:1 methanol:acetone. Images were acquired on a Zeiss LSM410 confocal microscope.

Immunoprecipitation. Cells were lysed (20 mM Tris–HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, plus phosphatase and protease inhibitors) at 4 °C for 1 h. Nuclei and insoluble material were removed by centrifugation. Antigen–antibody complexes were immunoprecipitated with 2 μg anti-PLIC or rabbit IgG and Protein-A–Sepharose (Amersham, UK) at 4 °C for 2 h. Beads were washed three times in lysis buffer and analysed by western blot as described previously (Bedford *et al*, 2001).

Glutathione *S*-transferase affinity chromatography. Transfected cells were lysed (20 mM Hepes, pH 7.4, 50 mM NaCl, 10% glycerol, 0.5% Triton X-100 plus protease inhibitors) at 4 °C for 1 h. Nuclei and insoluble material were removed by centrifugation. GST fusion proteins on glutathione–agarose (Sigma, St Louis, MO, USA), prepared as described previously (Bedford *et al*, 2001), were incubated with the lysates for 1 h, washed three times with buffer and analysed by western blot.

Semiquantitative reverse transcription–PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen). RT–PCR was carried out using 1 μg of complementary DNA. The PCR cycle number for each primer set was chosen on the basis of a preliminary study determining the linear range of amplification for each molecule. For PCR primers and further details, see the supplementary information online.

RNA interference. At 72 h and 24 h before fixation, HEK293 cells were transfected with 20 nM EPS15 (Huang *et al*, 2004), PLIC-1 (Massey *et al*, 2005) or scrambled short interfering RNA duplexes (Dharmacon, Lafayette, CO, USA) using HiPerFect (Qiagen, Hilden, Germany). In addition, at 24 h, cells were also transfected with 2.5 μg pAUI-Q78 using Lipofectamine 2000 (Invitrogen).

Cells were then processed for immunofluorescence as described above, and the number of cells with aggresomes was quantified. **Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank M. Cheetham, E. Fon, P. McPherson and G. Rouleau for constructs. This work was supported by the Canadian Institute of Research (CIHR). R.H. is a recipient of a CIHR Canada Graduate Scholarship Master's Award, and F.B. is a CIHR-Canada Research Chair—Tier II.

REFERENCES

Bedford FK *et al* (2001) $GABA_A$ receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein PLIC-1. *Nat Neurosci* **4:** 908–916

Bertram L *et al* (2005) Family-based association between Alzheimer's disease and variants in UBQLN1. *N Engl J Med* **352:** 884–894

Biggins S, Ivanovska I, Rose MD (1996) Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *J Cell Biol* **133:** 1331–1346

Burnett BG, Pittman RN (2005) The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation. *Proc Natl Acad Sci USA* **102:** 4330–4335

Chen L, Shinde U, Ortolan TG, Madura K (2001) Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly. *EMBO Rep* **2:** 933–938

Di Fiore PP, Polo S, Hofmann K (2003) When ubiquitin meets ubiquitin receptors: a signalling connection. *Nat Rev Mol Cell Biol* **4:** 491–497

Fisher RD, Wang B, Alam SL, Higginson DS, Robinson H, Sundquist WI, Hill CP (2003) Structure and ubiquitin binding of the ubiquitin-interacting motif. *J Biol Chem* **278:** 28976–28984

Hartmann-Petersen R, Gordon C (2004) Integral UBL domain proteins: a family of proteasome interacting proteins. *Semin Cell Dev Biol* **15:** 247–259

Hofmann K, Falquet L (2001) A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *Trends Biochem Sci* **26:** 347–350

Huang F, Khvorova A, Marshall W, Sorkin A (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* **279:** 16657–16661

Iwata A, Riley BE, Johnston JA, Kopito RR (2005) HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem* **280:** 40282–40292

Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* **143:** 1883–1898

Kleijnen MF, Shih AH, Zhou P, Kumar S, Soccio RE, Kedersha NL, Gill G, Howley PM (2000) The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. *Mol Cell* **6:** 409–419

Kleijnen MF, Alarcon RM, Howley PM (2003) The ubiquitin-associated domain of hPLIC-2 interacts with the proteasome. *Mol Biol Cell* **14:** 3868–3875

Layfield R, Lowe J, Bedford L (2005) The ubiquitin–proteasome system and neurodegenerative disorders. *Essays Biochem* **41:** 157–171

Mah AL, Perry G, Smith MA, Monteiro MJ (2000) Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation. *J Cell Biol* **151:** 847–862

Massey LK, Mah AL, Monteiro MJ (2005) Ubiquilin regulates presenilin endoproteolysis and modulates γ-secretase components, Pen-2 and nicastrin. *Biochem J* **391:** 513–525

Meriin AB *et al* (2003) Aggregation of expanded polyglutamine domain in yeast leads to defects in endocytosis. *Mol Cell Biol* **23:** 7554–7565

Novoa I, Zhang Y, Zeng H, Jungreis R, Harding HP, Ron D (2003) Stress-induced gene expression requires programmed recovery from translational repression. *EMBO J* **22:** 1180–1187

Pickart CM (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70:** 503–533

Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416:** 451–455

Regan-Klapisz E *et al* (2005) Ubiquilin recruits Eps15 into ubiquitin-rich cytoplasmic aggregates via a UIM–UBL interaction. *J Cell Sci* **118:** 4437–4450

Ross CA, Poirier MA (2004) Protein aggregation and neurodegenerative disease. *Nat Med* **10:** S10–S17

Toulouse A, Au-Yeung F, Gaspar C, Roussel J, Dion P, Rouleau GA (2005) Ribosomal frameshifting on MJD-1 transcripts with long CAG tracts. *Hum Mol Genet* **14:** 2649–2660

Walters KJ, Kleijnen MF, Goh AM, Wagner G, Howley PM (2002) Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. *Biochemistry* **41:** 1767–1777

Wang H, Lim PJ, Yin C, Rieckher M, Vogel BE, Monteiro MJ (2006) Suppression of polyglutamine-induced toxicity in cell and animal models of Huntington's disease by ubiquilin. *Hum Mol Genet* **15:** 1025–1041

Westhoff B, Chapple JP, van der Spuy J, Hohfeld J, Cheetham ME (2005) HSJ1 is a neuronal shuttling factor for the sorting of chaperone clients to the proteasome. *Curr Biol* **15:** 1058–1064

Wu AL, Wang J, Zheleznyak A, Brown EJ (1999) Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane. *Mol Cell* **4:** 619–625