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Ufd1–Npl4 is a negative regulator of cholera toxin retrotranslocation

Elizabeth McConnell, Agnieszka Lass, Cezary Wójcik *

Department of Anatomy and Cell Biology, Indiana University School of Medicine, 8600 University Blvd., Evansville, IN 47712, USA

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Abstract

The A1 chain of the cholera toxin (CT) undergoes retrotranslocation to the cytosol across the endoplasmic reticulum (ER) membrane by hijacking ER-associated degradation (ERAD). In the cytosol the CT A1 chain stimulates adenylyl cyclase. The $VCP^{Ufd1–Npl4}$ complex mediates retrotranslocation of emerging ER proteins. While one group reported that VCP is required for CT retrotranslocation, another group concluded the opposite. We show that VCP is dispensable for CT retrotranslocation, however RNAi of either Ufd1 or Npl4 induces an increase in adenylyl cyclase activity induced by CT. RNAi of VCP, Ufd1 or Npl4 did not affect adenylyl cyclase activity induced by forskolin. These findings are coherent with our previous report showing that depletion of Ufd1–Npl4 accelerates ERAD of reporter substrates. To integrate contradictory results we propose a new model, where Ufd1–Npl4 is a negative regulator of retrotranslocation, delaying the retrotranslocation of ERAD substrates independently of its association with VCP.

Keywords: Retrotranslocation; ER-associated degradation (ERAD); Valosin-containing protein (VCP); Cholera toxin; Adenylyl cyclase; Forskolin

Cholera toxin (CT) is produced by *Vibrio cholerae*, a Gram-negative bacterium causative of a severe water-borne disease associated with devastating diarrhoea [1,2]. It is composed of five B chains associated with an A chain, proteolytically cleaved into A1 and A2 chains held together by disulfide bridges. The B subunits bind to GM1 gangliosides on the external surface of the plasma membrane, what allows internalization and retrograde transport of CT towards the ER [3]. Once in the ER the subunits of CT dissociate mainly by the action of protein disulfide isomerase [4]. The A1 chain is then retrotranslocated through the Sec61 channel by a process requiring the ER chaperone BiP and ATP hydrolysis [5,6], which however does not require ubiquitination or proteasome activity [7]. Once in the cytosol, CT A1 chain rapidly refolds into an active enzyme, which diffuses to the inner side of the plasma membrane, where it ADP-ribosylates a trimeric G protein, which in turn activates adenylyl cyclase [2,8]. Increased cAMP levels stimulate opening of chloride channels at the plasma membrane, resulting in massive secretion of chloride and water, leading to diarrhoea in individuals infected with *V. cholerae* [1,2].

VCP (valosin-containing protein, p97; Cdc48 in yeast) is an abundant ATP-ase of the AAA family, which has been involved in multiple cellular functions, including ubiquitin- and proteasome-dependent degradation of proteins through the ERAD pathway [9]. An established model based on findings in yeast and on studies in reconstituted mammalian systems proposes that the VCP hexamer in association with the Ufd1–Npl4 heterodimer recognizes emerging ERAD substrates through a dual recognition mechanism, which involves binding of polyubiquitin chains by each of the elements of the complex as well as binding of VCP to misfolded portions of emerging polypeptides prior to their ubiquitination [10–13]. The involvement of Ufd1–Npl4 in ERAD is supported by the study of yeast Ufd1 and Npl4 mutants, which are defective in ERAD [14,15] and by the fact, that both Ufd1 and Npl4 are able to bind polyubiquitin chains [11,16]. However, at least some ERAD substrates may be retrotranslocated and degraded without the involvement of VCP [17,18]. We have recently

* Corresponding author. Fax: +1 812 465 1184.
E-mail address: cwojcik@iupui.edu (C. Wójcik).

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demonstrated that a >90% depletion of the $VCP^{Ufd1–Npl4}$ complex through RNAi of Ufd1 or Npl4 induces a paradoxical decrease in the levels of several established ERAD substrates [19].

Since CT A1 chain exploits the retrotranslocation machinery to reach the cytosol [8,12], the question whether VCP is involved in this process was addressed independently by two groups using a similar experimental approach [20,21]. The first group concluded that VCP is required for the retrotranslocation of CT A1 chain, since expression of dominant negative ATP-ase defective mutants of VCP reduced the adenylyl cyclase activity induced by CT and VCP coimmunoprecipitated with CT A1 chain [21]. The other group obtained similar results but came to an opposite conclusion, since overexpression of dominant negative ATP-ase completely inhibited the US11-dependent retrotranslocation of MHC class I heavy chains, while having only minimal effects on adenylyl cyclase activity induced by CT [20].

Expression of dominant negative mutants is a useful method to study the function of many proteins, however in case of VCP, a protein which has an inactive ATP-ase domain still may bind polyubiquitin chains and interact with other factors, including Ufd1–Npl4 [11]. In contrast, RNA interference (RNAi) allows a depletion of a protein to levels which depend on the requirement of a given protein for cell survival. In case of VCP it is possible to obtain a ~90% reduction of VCP before eliciting cell death [22]. Detailed microarray studies have demonstrated that this approach is very specific and does not elicit off target or interferon-like responses [18].

Materials and methods

HeLa cells were grown in Advanced DMEM (Invitrogen, Carlsbad, CA) supplemented with Gluta-MAX™, antibiotic/antimycotic solution and 2% fetal bovine serum (Gemini Bioproducts, Woodland, CA). Small interfering RNAs (siRNAs) were obtained by chemical synthesis using 2′-ACE chemistry from Dharmacon (Lafayette, CO). siRNAs were 2′ deprotected, desalted, purified by polyacrylamide gel electrophoresis, and duplexed by the manufacturer. The mass of each siRNA was verified by MALDI-TOF. After shipment in a dry form the siRNAs were suspended in the 1× universal buffer (20 mM KCl, 6 mM Hepes–KOH, pH 7.5, and 0.2 mM $MgCl_2$) at a 20 μM concentration, aliquoted, and frozen at −20 °C for further use. We used two siRNAs targeting VCP (VCP-2, positions 599–619; VCP-6, positions 480–500 of human VCP mRNA; Accession No. NM_007126), an siRNA targeting Ufd1 (positions 301–331 of human Ufd1 sequence; Accession No. AF141201), and an siRNA targeting Npl4 (positions 310–330 of human Npl4 sequence; Accession No. AK000664) as described previously [18,19,22]. RNAi was performed using X-tremeGENE™ (Roche Applied Science, Penzberg, Germany) as described previously [18,19]. Seventy-two hours after the transfection culture media were replaced with 0.5 ml of Hank's balanced salts solution with 0.3 mM isobutylmethylxanthine supplemented with either 1 μg/ml CT or 20 μM forskolin (all from Sigma, St. Louis, MO). Ten micromolar MG132 (Calbiochem) was used as a positive control of ERAD inhibition. After 4 h incubation the supernatants were collected and frozen at −80 °C while the cells were washed in PBS and lysed in RIPA buffer supplemented with Complete Mini™ protease inhibitors (Roche) and sodium orthovanadate (Sigma). Protein concentration was evaluated with the Bradford [23] assay (Bio-Rad, Hercules, CA) and samples were supplemented with Laemmli sample buffer to attain the same protein concentration (2 mg/ml) before running SDS–PAGE [24]. Western blot was performed using rabbit anti-actin antibody (Sigma), mouse anti-VCP antibody (BD Transduction Laboratories, Franklin Lakes, NJ), and custom made anti-Ufd1 serum described previously [19]. After incubation with HRP-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) reactions were developed using Advance™ ECL kit (Amersham Bioscience, Piscataway, NJ). Images were acquired using the Kodak4000MM imaging system (Kodak, Rochester, NY). cAMP levels in the supernatants were measured with the cAMP [^{125}I] radioimmunoassay kit (Perkin-Elmer, Boston, MA) using the LS6500 scintillation counter (BD, Franlin Lakes, NJ). Statistical analysis was performed using Microsoft Excel.

Results and discussion

Measurement of adenylyl cyclase activity after treatment of sensitive cells with CT is a standard method used to assay whether the A1 chain of CT has trafficked from the extracellular milieu to the ER and then through the retrotranslocation channel to reach adenylyl cyclase [20,21]. However, the overexpression of dominant negative mutants of VCP leads to ambiguous results suggesting a hypothetical direct inhibition of adenylyl cyclase [20]. To avoid the artifacts inherent to the dominant negative approach we have decided to deplete individual components of the $VCP^{Ufd1–Npl4}$ complex through RNAi. RNAi is a well-established method useful to study the function of gene products which are essential and whose knockout would have been lethal. We have successfully used it in the past to study the role of the $VCP^{Ufd1–Npl4}$ complex in different cellular processes, including degradation of cytosolic and ERAD substrates [18,19,22]. We have successfully depleted VCP and Ufd1 to ~10% of control levels (Fig. 1A). While the depletion of VCP and Ufd1 is shown directly by Western blotting with the respective antibodies, in case of Npl4 we observed an indirect effect of Ufd1 depletion, which results from the instability of the Ufd1 monomer in the absence of Npl4 [19]. As shown previously by microarray analysis, the RNAi procedure induces very few sequence-independent and/or off-target effects [18,19,22].

CT at 1 μg/ml induced within 4 h a fivefold increase in the adenylyl cyclase activity in untreated HeLa cells (Fig. 1B). Knockdown of VCP by two different siRNAs did not prevent this effect of CT confirming the conclusion that VCP is not involved in this process. However, we have not observed a decrease of the adenylyl cyclase activity induced by forskolin in contrast to Kothe et al. who reported that overexpression of a dominant negative ATP-ase mutant VCP decreases adenylyl cyclase activity [20]. Based on the current model of the role of $VCP^{Ufd1–Npl4}$ in retrotranslocation [12,13] we expected a similar negative result when either Ufd1 or Npl4 where depleted by RNAi. Unexpectedly, we observed a significant increase ($p < 0.008$) in the induction of adenylyl cyclase activity by CT in cells depleted from Ufd1 to Npl4. At the same time, forskolin activation of adenylyl cyclase in cells submitted to RNAi of Ufd1 or Npl4 was

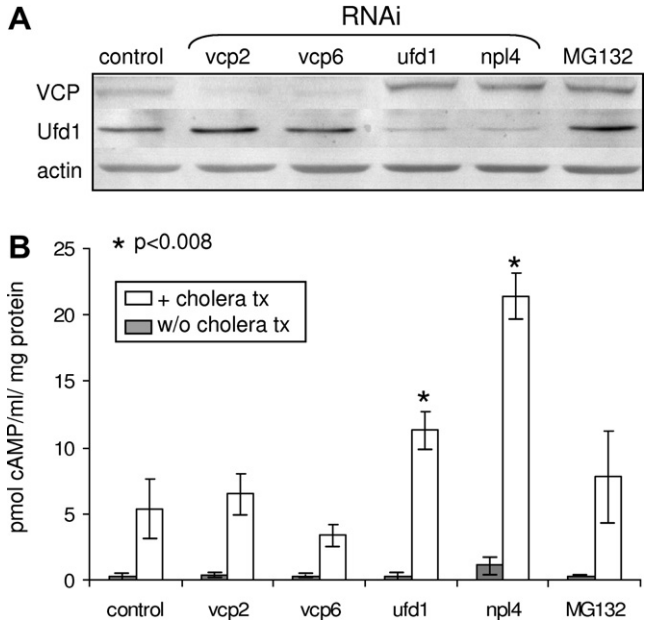

Fig. 1. RNAi of VCP using two different siRNAs (vcp2 and vcp6) does not affect the ability of cholera toxin to activate adenylyl cyclase, while RNAi of either Ufd1 or Npl4 induces a statistically significant ($p < 0.008$, Student's t-test) increase of the ability of cholera toxin (1 μg/ml) to stimulate adenylyl cyclase activity. (A) Western blotting demonstrates the reduction of VCP and Ufd1 levels, actin levels demonstrate equal protein loading. This is a typical blot from three different experiments. (B) Graphical representation of adenylyl cyclase activity normalized for protein concentration integrating data obtained from three independent experiments, each experiment having the measurements performed in duplicates.

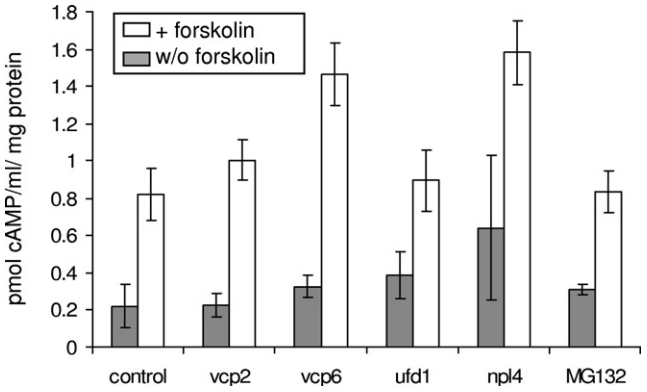

Fig. 2. RNAi of Ufd1, Npl4 or of VCP using two different siRNAs (vcp2 and vcp6) does not affect the ability of 20 μM forskolin to stimulate the adenylyl cyclase activity. Graphical representation of adenylyl cyclase activity normalized for protein concentration integrating data obtained from two independent experiments, each experiment having the measurements performed in duplicates. None of the observed differences is statistically significant ($p < 0.05$, Student's t-test).

unaffected. Since forskolin is a diterpene known to activate adelylyl cyclase, which does not use the retrotranslocation pathway [25], our results indicate that most likely the increased adenylyl cyclase activity reflects a facilitated transport of CT through the retrotranslocation pathway occurring in the absence of Ufd1–Npl4. Proteasome inhibitor MG132 did not affect the activity of adenylyl cyclase indicating that in contrast to ERAD substrates CT is not degraded following retrotranslocation in accordance with other reports [7] (Fig. 2).

Our experiments suggest that CT A1 chain is retrotranslocated to the cytosol without a direct involvement of VCP as proposed by Kothe et al. [20]. The driving force for the retrotranslocation of CT is likely provided by the ER chaperone BiP [6]. However, we also provide evidence that the Ufd1–Npl4 complex delays retrotranslocation of CT, which appears to be more efficient and/or proceeds faster when Ufd1–Npl4 is depleted. This result cannot be explained with the current model of the role performed by $VCP^{Ufd1–Npl4}$ during retrotranslocation [13,26]. We therefore propose a new model, which reconcile our findings obtained with the use of RNAi with the findings obtained through the use of dominant negative VCP mutants. We propose that the Ufd1–Npl4 heterodimer performs in mammalian cells the role of a "gatekeeper" at the cytoplasmic surface of the retrotranslocation channel. According to this new model, Ufd1–Npl4 binds to polypeptides emerging through the retrotranslocation channel, preventing their immediate transfer to the cytosol, which may be propelled by BiP on the ER side of the channel. Active VCP bound to Ufd1–Npl4 performs ATP hydrolysis which is necessary to relieve the Ufd1–Npl4-mediated inhibition of retrotranslocation and probably to transfer the substrate to the 26S proteasome for degradation with a possible aid of additional factors recruited by VCP [27]. Thus, overexpression of an ATPase defective VCP, which is however still able to bind to the Ufd1–Npl4 dimer [11], creates a situation, where Ufd1–Npl4 attached to the emerging polypeptide chain delays its retrotranslocation since the mutant VCP cannot relieve this block. Such delay of retrotranslocation of an ERAD substrate results in its accumulation within the ER, while in case of CT increased retention in the ER leads to the observed decrease in adenylyl cyclase activity [20,21]. On the other hand, depletion of Ufd1–Npl4 through RNAi of either one of those proteins removes the occlusion on the cytoplasmic side of the retrotranslocation channel allowing a faster efflux of ERAD substrates [19] and CT, in the latter case leading to increased adenylyl cyclase activity as observed here. Lack of effects of VCP depletion suggests that VCP is not necessary for retrotranslocation of CT A1 chain and the role of Ufd1–Npl4 in this process; indeed, VCP is required for the retrotranslocation of some (αTCR, MHC class I heavy chains) but not all (δCD3, α1-antitrypsin) emerging substrates [10,18]. Our model reconciles seemingly contradictory findings from different groups much better than the current model of the role of the $VCP^{Ufd1–Npl4}$ complex in retrotranslocation [12,13]. It raises new questions open for experimental verification. In particular it predicts that Ufd1–Npl4 will bind to emerging substrates in the absence of VCP or in the presence of inactive VCP, which may be required to provide a scaffold for this complex to

function properly. In case of polyubiquitinated substrates such binding is perfectly possible to occur independently of VCP [16], however in case of CT, which does not undergo polyubiquitination [7], other mechanisms must be involved.

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