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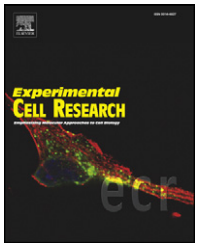

Research Article

Recruitment of APPL1 to ubiquitin-rich aggresomes in response to proteasomal impairment

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ABSTRACT

Inhibitors of proteasomes have been shown to affect endocytosis of multiple membrane receptors, in particular at the step of cargo sorting for lysosomal degradation. Here we demonstrate that the inhibition of proteasomes causes specific redistribution of an endosomal adaptor APPL1, which undergoes initial solubilization from APPL endosomes followed by clustering in the perinuclear region. MG132 treatment decreases APPL1 labeling of endosomes while the staining of the canonical early endosomes with EEA1 remains unaffected. Upon prolonged treatment with proteasome inhibitors, endogenous APPL1 localizes to the site of aggresome formation, with perinuclear APPL1 clusters encapsulated within a vimentin cage and co-localizing with aggregates positive for ubiquitin. The clustering of APPL1 is concomitant with increased ubiquitination and decreased solubility of this protein. We determined that the ubiquitin ligase Nedd4 enhances polyubiquitination of APPL1, and the ubiquitin molecules attached to APPL1 are linked through lysine-63. Taken together, these results add APPL1 to only a handful of endogenous cellular proteins known to be recruited to aggresomes induced by proteasomal stress. Moreover, our studies suggest that the proteasome inhibitors that are already in clinical use affect the localization, ubiquitination and solubility of APPL1.

Introduction

Receptor-mediated endocytosis is a process by which cells internalize extracellular ligands. It is initiated by the inward budding of plasma membrane vesicles containing ligands bound to specific transmembrane receptors. Among other routes, receptors can be internalized via clathrin-coated pits that pinch off, lose their clathrin coat and fuse with an endosomal compartment where the cargo is sorted towards recycling or degradation in lysosomes. Several endosomal populations can be distinguished based on the presence of specific markers as well as functional and morphological characteristics. Early endosomes that contain the small GTPase Rab5 and EEA1 (early endosome antigen 1) are the first cargo sorting platform [1]. A subpopulation of early endosomes, initially characterized by the presence of APPL1 protein as a unique marker [2], are called APPL1-positive vesicles or APPL endosomes [3]. From early endosomes various types of cargo are transported to recycling endosomes, late endosomes and lysosomes, or towards the Golgi apparatus.

Ubiquitination acts as a signal for the internalization and sorting of plasma membrane proteins along the endocytic route (reviewed in [4]). In contrast to the well-established function of polyubiquitination in proteasome-dependent protein degradation, the ubiquitination involved in endocytosis does not lead to protein destruction

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Abbreviations: APPL1, adaptor protein containing PH domain, PTB domain and leucine zipper motif; EEA1, early endosome antigen 1; GFP, green fluorescent protein; HA, hemagglutinin; MIP, maximal intensity projection

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in proteasomes. Still, during endocytic trafficking of epidermal growth factor (EGF) receptor there are several stages sensitive to the inhibitors of proteasome, namely the sorting from early to late endosomes and the translocation of activated EGF receptor from the outer limiting membrane to the inner membranes of multivesicular bodies (MVBs) [5,6]. Ubiquitin-dependent sorting is specific to endocytosed cargo, as proteasomal inhibitors block ligand-induced internalization of glutamate receptors, but not of transferrin receptor [7]. In general, inhibition of proteasomes blocks the sorting of membrane receptors towards degradation without interfering with the transport of soluble proteins or recycling cargo [8]. The application of proteasome inhibitors decreases the rates of lysosomal degradation of multiple transmembrane receptors, including those for EGF [9], growth hormone and nerve growth factor [8], platelet-derived growth factor [10], hepatocyte growth factor [11], interleukin-2 [12] and low-density lipoprotein receptor-related protein [7].

In contrast to the general knowledge about the involvement of ubiquitin–proteasome system in endocytosis, specific effects of proteasome inhibitors on early endosomes are not known. In particular, we were interested in the fate of the subpopulation of early endosomes marked by the presence of APPL1, which act as a sorting and signaling platform. APPL endosomes are positive for Rab5 but not EEA1 (which is a marker for the canonical early endosomes), and are preferentially localized underneath the plasma membrane [2]. APPL1 is primarily recruited to endocytic membranes through binding to the small GTPase Rab5. The endocytic compartments are highly dynamic, and changes in the composition of phosphoinositides on early endocytic membranes cause a selective recruitment of either APPL1 or EEA1 to Rab5-positive endosomal vesicles [3]. APPL endosomes participate in endocytic trafficking of EGF receptor [2] and transferrin receptor (our unpublished results). In addition, they function as platforms for the assembly of signaling complexes that control distinct signal transduction pathways, e.g. the Akt and MAPK pathways [3,13]. APPL1, a unique marker of APPL endosomes, interacts with Akt and phosphatidylinositol 3-kinase, as well as with several membrane receptors (including adiponectin receptor 1 and 2, netrin-1 receptor DCC, follicle stimulating hormone receptor, and nerve growth factor receptor TrkA), contributing to signal transduction downstream of these receptors (reviewed in [14]). The cellular functions of APPL1 are not limited to endocytosis, and include also regulation of gene transcription, metabolism, cell proliferation and cell survival [14]. Recently, we reported that APPL1 can activate β-catenin-dependent transcription [15] and interact with the NuRD chromatin remodeling and histone deacetylase complex [2,16].

While proteasomal inhibition affects endocytic trafficking already upon short application (1 h or less), prolonged (12–24 h) treatment causes massive changes in the turnover of cellular proteins. Proteasome deficiency stimulates a cellular stress response by inducing time- and dose-dependent inhibition of cell growth, arrest of the cell cycle, apoptosis, as well as the loss of mitochondrial membrane potential and increase in the intracellular ROS levels [17]. Compromised proteasomal function leads to the accumulation of misfolded proteins which become sequestered into large, insoluble, non-membranous protein deposits called aggresomes. These structures were first described by Wojcik et al. in HeLa cells treated with a peptide aldehyde proteasomal inhibitor PSI [18], and named aggresomes by Johnston et al. [19]. Aggresomes appear either as a single sphere with a diameter of 1–3 μm positioned at an indentation of the nucleus, or as an extended ribbon around the nuclear envelope [20]. Aggresomes assemble in the perinuclear region near the microtubule organizing center, and their formation involves trafficking of aggregated proteins along microtubules and reorganization of intermediate filaments [19–21]. Aggresomes recruit chaperones, ubiquitination enzymes and components of the ubiquitin–proteasome machinery, to help in the disposal of aggregated proteins. The process of aggresome clearance is largely dependent on the autophagy–lysosome system. The cellular site of aggresome formation is enriched in double-membrane vesicles representing autophagosomes, and blockage of autophagy impairs the clearance of aggresomes [22]. Lysosomes gather close to the aggresome and eventually digest the proteins forming the inclusions.

Here, we aimed to characterize an effect of proteasomal inhibition on APPL endosomes. We analyzed the localization and morphometric features of APPL1-positive endosomes upon a range of incubation times with proteasome inhibitors. Strikingly, we observed initial solubilization of APPL1 from endosomal membranes followed by its relocalization to aggresomes which correlated with increased ubiquitination and insolubility of APPL1.

Materials and methods

Antibodies and chemicals

Anti-APPL1 polyclonal antibodies against C-terminal peptides were raised in rabbits (Eurogentech) and previously described [2,15]. The following mouse monoclonal antibodies were used for immunofluorescence: anti-EEA1 and GM130 (BD Transduction Laboratories), CD63 (Developmental Studies Hybridoma Bank), Rab5 (D-11, Santa Cruz Biotechnology), ubiquitin (FK1, Biomol), vimentin and GFP (Sigma-Aldrich). Alexa Fluor 405-, 488- and 555-conjugated anti-mouse and anti-rabbit antibodies, as well as EGF and transferrin labeled with Alexa Fluor 488 were from Invitrogen. Additional antibodies were used in Western blotting: mouse antibodies against HA and ubiquitin P4D1 (Santa Cruz Biotechnology), α-tubulin, β-actin and FLAG (M2) (Sigma-Aldrich), HDAC2 (Upstate), and goat antibody against GFP (MPI, Dresden). Secondary horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch. Mito-Tracker Orange probe (Invitrogen) was used according to provider's instructions. Cycloheximide, 4′,6-diamidino-2-phenylindole (DAPI), N-ethylmaleimide (NEM) and nocodazole were purchased from Sigma-Aldrich. Proteasome inhibitors MG132, ALLN and clasto-lactacystin β-lactone were from Sigma-Aldrich, and bortezomib from LC Laboratories. The inhibitors were diluted in dimethyl sulfoxide (DMSO) and an equivalent volume of DMSO was used in all experiments as a solvent control.

Plasmids and siRNA reagents

The constructs of untagged, Myc-tagged and GFP-tagged APPL1 were previously described [2]. The expression constructs of FLAG- and HA-tagged ubiquitin wild type, HA-tagged ubiquitin mutant K63R, HA-tagged c-Cbl and untagged Nedd4 were a gift from Ivan Dikic. The HA-ubiquitin K63-only expression construct was provided by Ted M. Dawson (Addgene plasmid 17606) [23]. The mouse HA-Nedd4-1 expression construct was provided by Allan M. Weissman (Addgene plasmid 11426) [24]. The mouse GFP-Eps15 construct was previously described [25].