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THE KDEL RECEPTOR INDUCES AUTOPHAGY TO PROMOTE THE CLEARANCE OF NEURODEGENERATIVE DISEASE-RELATED PROTEINS

P. WANG, B. LI, L. ZHOU, E. FEI* AND G. WANG*

Laboratory of Molecular Neuropathology, CAS Key Laboratory of Brain Function and Disease, and School of Life Sciences, University of Science & Technology of China, Chinese Academy of Sciences, Hefei, Anhui 230027, PR China

Abstract—Endoplasmic reticulum (ER) stress is involved in neurodegenerative diseases, and the KDEL (Lys-Asp-Glu-Leu motif) receptor (KDELR) plays a key role in ER quality control and in the ER stress response. The subcellular distribution of KDELR is dynamic and related to its ligand binding status and its expression level. Here, we show that KDELR mRNA is upregulated upon thapsigargin treatment, which induces ER stress. Moreover, overexpressed KDELR partially redistributes to the lysosome and activates autophagy. The R169N mutant, a ligand binding-defective form of KDELR, and D193N, a transport-defective form of KDELR, both fail to trigger autophagy. Overexpression of KDELR activates extracellular signal-regulated kinases (ERKs). Both the activation of ERKs and autophagy induced by KDELR could be blocked by PD98059, an inhibitor of mitogen extracellular kinase 1 (MEK1). The overexpression of some neurodegenerative disease-related proteins, such as amyotrophic lateral sclerosis (ALS)-linked G93A superoxide dismutase 1 (SOD1), Parkinson's disease-associated A53T alpha-synuclein and Huntington's disease-related expanded huntingtin, increase the mRNA levels of KDELR. Moreover, the overexpressed KDELR promotes the clearance of these disease proteins through autophagy. Taken together, our data provide evidence that KDELR, as a novel inducer of autophagy, participates in the degradation of misfolded neurodegenerative disease-related proteins.

Key words: KDEL receptor, ER stress, neurodegenerative disease-related proteins, autophagy.

*Corresponding author. Tel: +86-551-3607058; fax: +86-551-3607058.
E-mail address: wghui@ustc.edu.cn (G. Wang) or ericfee@ustc.edu.cn (E. Fei).

Abbreviations: ALS, amyotrophic lateral sclerosis; ARF1, ADP ribosylation factor 1; ARF1-GAP, ARF1 GTPase-activating protein; ATG5, autophagy-related gene 5; BIP, binding immunoglobulin protein; CHOP, CAAT/enhancer binding protein homologous protein; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescence protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERGIC, ER-golgi compartments; ERKs, extracellular signal-regulated kinases; HD, Huntington's disease; Htt, huntingtin; JNKs, c-Jun amino-terminal kinases; KDEL, Lys-Asp-Glu-Leu; KDELR, KDEL receptor; KO, knockout; LC3, microtubule-associated protein 1 light chain 3; MAPKs, mitogen-activated protein kinases; MEK1, mitogen extracellular kinase 1; PBS, phosphate buffered saline; PD, Parkinson's disease; RFP, red fluorescence protein; SOD1, superoxide dismutase 1; TEM, transmission electron microscopy; TG, thapsigargin; WT, wild-type; α-syn, alpha-synuclein.

The endoplasmic reticulum (ER) is the major organelle responsible for protein synthesis, folding, post-translational modification and the transport of nascent proteins to different cellular targets (Lindholm et al., 2006). Protein quality control in the ER is important to ensure the fidelity of protein synthesis and folding. The accumulation of unfolded proteins induces ER stress, which can cause neuronal cell death in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), polyglutamine diseases, and prion diseases (Lindholm et al., 2006; Scheper and Hoozemans, 2009). The abnormal accumulation of misfolded proteins is a pathological hallmark of many neurodegenerative diseases (Ross and Poirier, 2004). The accurate elimination of these disease proteins through quality control mechanisms is important for ER homeostasis and is closely associated with the pathogenesis of these diseases (Scheper and Hoozemans, 2009). Many neurodegenerative disease-related proteins are localized to the ER and are involved in ER stress. For instance, A53T alpha-synuclein, a PD-related pathogenic alpha-synuclein (Webb et al., 2003; Smith et al., 2005; Hoozemans et al., 2007; Bellucci et al., 2011), and the polyglutamine expanded huntingtin, a Huntington's disease (HD)-related protein, both induce ER stress (Duennwald and Lindquist, 2008; Reijonen et al., 2008; Carnemolla et al., 2009). ALS-linked mutant superoxide dismutase 1 (SOD1) accumulates in the ER and results in ER stress (Atkin et al., 2006; Nishitoh et al., 2008; Oh et al., 2008; Ying et al., 2009; Li et al., 2010).

The KDEL (Lys-Asp-Glu-Leu motif) receptor (KDELR), a human homologue of the yeast HDEL (His-Asp-Glu-Leu motif) receptor, was reported to be involved in ER stress and ER quality control (Yamamoto et al., 2001, 2003). KDELR usually recognizes proteins that contain a KDEL motif and promotes their retrograde transport from the Golgi complex to the ER (Lewis and Pelham, 1990, 1992a,b). Notably, ER-retained chaperones, such as calnexin, binding immunoglobulin protein (BIP), and protein disulfide isomerase (PDI), are among the retrieved KDEL-containing proteins, and are responsible for the modification and assembly of newly synthesized or misfolded proteins, processes which are important for the ER response against cellular stress (Yamamoto et al., 2003). Retrotransport of these critical chaperones promotes the retrieval of misfolded proteins back to the ER for refolding or ER-associated degradation (ERAD), thus making a vital contribution to ER quality control (Yamamoto et al., 2001).

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KDELR is primarily localized to the Golgi, ER, and the intermediate ER-Golgi compartments (ERGIC) (Lewis and Pelham, 1992a; Griffiths et al., 1994). At the steady-state level, KDELR is mainly concentrated in the perinuclear Golgi complex, but it is redistributed from the Golgi to the ER when it is activated by ligand binding (Hsu et al., 1992; Lewis and Pelham, 1992a; Tang et al., 1993). Increased KDELR expression induces its autoactivation and redistribution in a reticular, ER-like pattern (Hsu et al., 1992; Tang et al., 1993; Griffiths et al., 1994). Under stressful conditions, such as virus infection or heavy traffic loads, the localization of KDELR also changes (Hsu et al., 1992). The alteration of the subcellular distribution of KDELR implies that it may have additional functions under specific conditions. Here, we demonstrate a novel function for KDELR in association with autophagy and the clearance of neurodegenerative disease-related proteins.

EXPERIMENTAL PROCEDURES

Plasmid construction

The Myc-tagged full-length KDELR plasmids were kindly shared by Dr. Victor Hsu (Brigham and Women's Hospital/Harvard Medical School, Boston, MA, USA). KDELR cDNA was amplified by PCR with the primers 5′-GCAGGATCCCCATGAATCTCTTCCGATTCCTGG-3′ and 5′-GATCTCGAGTGCCGGCAAACTCAACTTCTTCCC-3′ and then inserted in-frame into pEGFP-N3 (Clontech Laboratories, Palo Alto, CA, USA) and p3xFlag-CMV-myc-24 (Sigma, St. Louis, MO, USA) plasmids. The plasmid pKH3-KDELR was obtained by excising KDELR cDNA from p3xFlag-CMV-myc-24-KDELR at HindIII/BamHI sites and inserting this fragment in-frame into pKH3 (Addgene, Cambridge, MA, USA). Two KDELR mutants, D193N and R169N, were constructed using a site-directed mutagenesis kit (Takara, Otsu, Shiga, Japan) with the primers 5′-TGCAATTTCTTCTACCTCTAT-3′ and 5′-GTAGAGGACTGTCTGGAC-3′ for D193N and 5′-TGGAACTACCATTTCGAGGGC-3′ and 5′-GATCCAGTTGAAGAGATAGAG-3′ for R169N. The plasmids pEGFP-N3-p62, pEGFP- and pDsRed-LC3 were described previously (Ren et al., 2010). Enhanced green fluorescence protein (EGFP)-fused G93A SOD1, A53T alpha-synuclein and $tHttQ_{150}$ (truncated huntingtin with 150 glutamines) constructs were described previously (Wang et al., 1999; Ying et al., 2009; Li et al., 2010). The fidelity of all constructs was confirmed by sequencing.

Cell culture, transfection and drug treatments

Autophagy related gene 5 (*ATG5*) wild-type (WT) and knockout (KO) MEF cells were kindly shared by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). H1299 cells, HEK293A cells, N2a cells, HeLa and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Los Angeles, CA, USA) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) with 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen Carlsbad, CA, USA). Stable cell lines were established using limiting dilutions with 200 μg/ml of G418 (Invitrogen) and screened by immunoblotting or fluorescent microscopy.

For transfection, cultured cells were washed with phosphate buffered saline (PBS) buffer and then transfected with suitable plasmids using Lipofectamine 2000 (Invitrogen) in DMEM without serum. DMEM containing 10% FBS was added to the culture medium 6 h after transfection. Cells were processed for further analysis at 24 h or 48 h after transfection.

For drug treatments, HeLa cells were treated with thapsigargin (TG) (Sigma) at a working concentration of 40 nmol/L. Cells were harvested for total RNA extraction 24 h later. In kinase inhibitor experiments, cells were treated with 20 μmol/L PD98059 (Cell Signaling Technology, Beverly, MA, USA), 10 μmol/L SP600125 (Calbiochem, San Diego, CA, USA) or 10 μmol/L SB203580 (Promega, Madison, WI, USA) 12 h after plasmid transfection and were collected 24 h later for immunoblot analysis.

KDELR siRNA knockdown

Oligonucleotides targeting KDELR were synthesized by GenePharma (Shanghai, China) with the sequences sense 5′-CCACGGUCUGGUUGAUUUATT-3′ and antisense 5′-UAAAUCAACCAGACCGUGGTG-3′. Meanwhile, a nonspecific control siRNA (si-NC) served as a negative control. The oligonucleotides were transfected with Oligofectamine (Invitrogen) following the instructions of the manufacturer.

Immunofluorescence

Cells were washed with PBS (pH 7.4) and then fixed with 4% paraformaldehyde for 5 min at room temperature. The fixed cells were incubated with PBS containing 0.25% Triton X-100 for 10 min, then blocked with PBS containing 4% fetal bovine serum. Cells were incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C followed by an incubation with rhodamine-conjugated sheep anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Finally, cells were observed using an inverted IX71 microscope system (Olympus, Tokyo, Japan).

Transmission electron microscopy

Cells were fixed in suspension with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) overnight at 4 °C and then post-fixed with 2% glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.4) for 1 h at room temperature. After dehydration with an ethanol series and propylene oxide, cells were embedded in Epon. Thin sections were stained with Uranyl Acetate and Lead Citrate and observed using a transmission electron microscope (JEOL-1230, Tokyo, Japan).

Immunoblot analysis and antibodies

Cells were lysed in RIPA lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Approximately 20 μg cell lysate was separated on a 12% or 15% SDS-PAGE gel and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Immunoblot analysis was carried out with the following primary antibodies: mouse monoclonal antibodies against GAPDH (Chemicon, Temecula, CA, USA), HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MEK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p62 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), SAPK/JNKs (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-tubulin (Sigma), phospho-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and phospho-SAPK/JNKs (Cell Signaling Technology) and rabbit polyclonal antibodies against ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), LC3 (Novus Biologicals, Littleton, CO, USA), phospho-MEK1 (Cell Signaling Technology), and phospho-p38 (Cell Signaling Technology). Sheep anti-mouse or anti-rabbit IgG-HRP secondary antibodies (Amersham Pharmacia Biotech, Pis-