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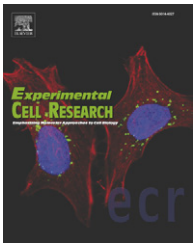

Research Article

Dual degradation mechanisms ensure disposal of NHE6 mutant protein associated with neurological disease

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ABSTRACT

Clinical features characterizing Angelman syndrome, previously shown to be caused by disruption of *UBE3A*, were recently also described in neurologically disabled patients with mutations in *SLC9A6*, which encodes the Na^+/H^+ exchanger NHE6. In the present work we have focused on NHE6Δ255–256, the protein product of a specific 6-bp patient deletion in *SLC9A6*. To resolve the molecular mechanism causing the cellular dysfunction associated with this mutant, we have characterized its intracellular behaviour in comparison to wild type NHE6. Our study demonstrates that NHE6Δ255–256 is much less stable than the wild type protein. Whereas wild type NHE6 is transported to the plasma membrane and early endosomes and remains stable, NHE6Δ255–256 is degraded via two independent pathways mediated by proteasomes and lysosomes, respectively. Depletion of NHE6 had no detectable effect on endosomal pH, but co-depletion of NHE6 and the closely related NHE9 caused enhanced acidification of early endosomes. Our results suggest that NHE6 participates in regulation of endosomal pH and provides a cellular basis for understanding the loss of NHE6 function leading to a neurological phenotype resembling Angelman syndrome.

Introduction

Angelman syndrome is a neurodevelopmental disorder characterized by symptoms such as severe developmental delay, hyperkinetic movement disorder, a happy demeanour, speech impairment and epileptic seizures [1]. The genetic basis for the disorder varies from large interstitial maternally inherited deletions of chromosome 15q11–13, to alterations in imprinting and point mutations. In spite of this, all identified mechanisms involve disturbance of the gene *UBE3A*. The protein product of the *UBE3A* gene is the E3-ubiquitin ligase E6-AP, which was first identified as a mediator of ubiquitination and degradation of p53 in association with the E6 oncoprotein of the human papilloma virus [2,3]. Apart from the virus-mediated p53 ubiquitination by E6-AP, the role of this ligase has remained elusive. *UBE3A* is maternally imprinted in the brain, particularly in the hippocampus and cerebellum, and studies in

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Abbreviations: aa, amino acid; EEA1, early-endosomal antigen 1; EGF, epidermal growth factor; EGFR, EGF receptor; ESCRT, endosomal sorting complex required for transport; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GFP, green fluorescent protein; NHE, Na+/H+ exchanger; wt, wild type; bp, base pair; UBE3A, ubiquitin protein ligase E3A; SLC9A6, solute carrier family 9, member 6

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mice with a maternal null mutation and Angelman syndrome-related features have revealed defects in the neuronal process of long-term potentiation (LTP) [4] and abnormal dendritic spine development [5]. Moreover, these mice have reduced activity of calcium/calmodulin-dependent kinase type 2 (CaMKII) in the brain by inhibitory phosphorylation [6], and the Angelman syndrome-like features can be rescued by introducing a mutation preventing this inhibitory phosphorylation [7].

Recently, a 6-bp deletion in the gene *SLC9A6* was identified as the causative agent in an X-linked mental retardation family displaying seizures, ataxia and other symptoms resembling those of Angelman syndrome [8]. Subsequently, three other mutations in the gene were identified in patients with genetically unexplained Angelman syndrome-like phenotypes, including a large pedigree designated as having Christianson syndrome [43]. *SLC9A6* encodes NHE6, a member of the Na^+/H^+ exchanger (NHE) family that mediates the electroneutral exchange of H^+ for Na^+ or K^+ across membranes. NHE6 localizes to early/recycling endosomes, and has been hypothesized to regulate endosomal pH by exporting H^+ out of the endosome lumen [9]. The endocytic pathway is characterized by a luminal acidified pH gradient, ranging from the neutral pH of newly internalized vesicles to pH 5.0 and lower in lysosomes, and this pH gradient is essential for many transport processes. The yeast homologue of NHE6, Nhx1, regulates endosomal pH and trafficking, and a direct link between the pH regulation and the trafficking function has been demonstrated [10,11]. Apart from the endosomal localization, NHE6 has been found to transiently localize to the plasma membrane [9], mediated by an interaction with Rack1 [12].

The identified 6-bp mutation in *SLC9A6* results in deletion of amino acids 255–256 of the NHE6 protein. These two residues are situated in a highly conserved region of the predicted transmembrane domain 7 of NHE6, and Glutamate 255 is essential for the ion transport in the NHE family members NHE1 and NHE8 [13–15]. In order to understand the molecular basis for the protein dysfunction caused by the 2-aa deletion, we have characterized the cellular behaviour of the deletion protein. Our results indicate that NHE6Δ255–256 is unstable in the cell and is rapidly degraded by two independent mechanisms; proteasomal degradation and lysosomal degradation. Moreover, we present evidence supporting the hypothesis that NHE6 and the closely related NHE9 are involved in regulation of endosomal pH.

Material and methods

Plasmid constructs and siRNA

The NHE6 construct (NHE6.0 isoform b) containing a GFP and a HA tag was a kind gift from Dr. R. Rao (The John Hopkins University School of Medicine, Baltimore, USA). The NHE6Δ255–256-GFP was generated from NHE6-GFP by quickchange mutagenesis PCR introducing the 6 bp deletion (nucleotide 764–769 in NHE6.0 ORF) and a silent restriction enzyme site for construct verification using the primer sequence: 5′-GCACTTCTTTTTGGGGTCCTCAATGATGCTG-3′. A pcDNA3.1 hemagglutinin epitope (HA) tagged K44A mutant construct of dynamin 1 was a generous gift from Dr. S. L. Schmid (The Scripps Research Institute, La Jolla, CA). The pcDNA3-myc-Ubiquitin construct has been previously described [16]. The single siRNA oligo used for depletion of Hrs has been described previously [17], and for depletion of Tsg101 a single siRNA oligo with the target sequence CCGTTTAGATCAAGAAGTATT was used. A nontargeting siRNA oligo from Dharmacon was used as a negative control, and the knock down efficiency was assessed by Western analysis. The siRNA oligos used to knock down the expression of NHE6 and NHE9 were the ON-TARGETplus SMARTpools with upgrade of single oligos from Dharmacon (NHE6: L-007626-00, sense sequences: I-CAGCCTAAGCTTACTAATATT, II-CGAGTGATGTAAATAATGTTT, III-GAAACCGGCCTGGCTATGATT, IV-GAACTGGTCATTCGAGGAATT, NHE9: L-007335-00, sense sequences: I-GATAGTTGCTGTTCTCTTCTT, II-GAGTATCAGTTTCAACATCTT, III-GGAAGCAAATAACTTGGATTT, IV-CATGCAGGATATAGTCTAATT). A nontargeting SMARTpool or single oligo was used as a negative control, and the knock down efficiency was assessed by relative real-time PCR quantification.

Confocal immunofluorescence microscopy

HeLa cells grown on coverslips were permeabilized with 0.05% saponin, fixed with 3% paraformaldehyde, and stained for fluorescence microscopy as described [18]. Coverslips were analyzed with a Zeiss LSM 510 META confocal microscope equipped with a Plan-Apochromat X63/1.4 and Neo-Fluar X100/1.45 oil-immersion objectives. Appropriate emission filter settings were included to exclude bleed-through effects. Either random cells expressing NHE6 (Fig. 2 and Fig. 3) or random cells with vesicular localization of expressed NHE6 (Fig. 4, Fig. 5 and Fig. 6) were scanned at intensity settings below saturation. The percentage of colocalization between two fluorochromes (defined as overlapping pixels) was determined using the histogram function of the Zeiss LSM 510 Software (version 3.2), where all pixel values above the background level were quantified. A minimum of 30 cells per sample and time point were quantitated and all experiments were repeated with comparable results. Images were processed with Photoshop CS2 (version 9.0).

Cell culture and transfection

HeLa cell cultures were maintained as described by ATCC. pEGFP or pcDNA3 constructs were transfected into cells using FuGENE 6™ (Roche Diagnostics) or Effectene™ (Qiagen Inc.) according to the manufacturer's instructions. Transfection of HeLa cells with siRNA oligonucleotides (25–100 nM) was performed as previously described [19] using Oligofectamine™ (Invitrogen) or Lipofectamine RNAi MAX™ (Invitrogen) and according to the manufacturer's instructions.

Antibodies

Rabbit antibodies against recombinant Hrs have been described before [20]. Human anti-early-endosomal antigen (EEA)1 antiserum [21] was a gift from Ban-Hock Toh (Monash University, Melbourne, Australia). The rabbit anti-lysosomal-associated membrane protein (Lamp)1 antibody was obtained from Sigma-Aldrich and the mouse monoclonal anti-Tsg101 from Abcam. The anti-GFP antibody used for immunofluorescence was obtained from Clontech and antiserum against GFP used for immunoprecipitation was kindly provided by Terje Johansen (Tromsø University, Norway). The mouse monoclonal antibody recognizing conjugated mono- and polyubiquitin (FK2) from Affiniti Research Products