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Overexpression of mouse GlcNAc-1-phosphotransferase-γ subunit in cells induced an I-cell-like phenotype of mucolipidosis

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Abstract

In a screen of signal peptide-containing proteins from a mouse hypothetical protein library, we identified the mouse UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase-γ chain (GlcNAc-1-phosphotransferase-γ) (GenBank accession no. AAR19081, HYP36 in this study). The mouse GlcNAc-1-phosphotransferase-γ was localized in the Golgi complex in cells and was expressed ubiquitously in mouse tissues, as shown by fluorescence microscope analysis and a semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) assay, respectively. Domain analysis showed that the mouse GlcNAc-1-phosphotransferase-γ had a conserved mannose-6-phosphate (M-6-P)-binding domain. Interestingly, we found that overexpression of the mouse GlcNAc-1-phosphotransferase-γ in fibroblast cell line NIH-3T3 induced accumulation of macromolecules, formation of large cytoplasmic vacuoles and decrease of lysosomal enzymes in cells. This phenotype was reminiscent of inclusion cells (I-cells) that were reported in mucolipidosis diseases caused by abnormal sorting of lysosomal proteins. Transient ectopic expression of GlcNAc-1-phosphotransferase-γ in endoplasmic reticulum (ER) induced lowered lysosomal enzyme activity in cells. These results suggested on one hand that GlcNAc-1-phosphotransferase-γ is an essential subunit of the GlcNAc-1-phosphotransferase, and on the other hand, the molecule might not only recognize the substrates of GlcNAc-1-phosphotransferase, but also the lysosomal proteins with M-6-P residuals.

Keywords: I-cells; M-6-P; Protein sorting; Lysosomal enzymes; Gene therapy

1. Introduction

Lysosomes are organelles responsible for clearance of accumulated macromolecules including glycogens, lipids and so on, in cells. The main components of lysosomes are therefore enzymes catalyzing the degradation of these molecules, such as glycosidases, lipases, etc. These enzymes, as well as other lysosomal proteins, are sorted to lysosomes mainly by two kinds of protein-sorting signals. One is a signal peptide on the N-terminal of newly synthesized lysosomal proteins, which targets the newly synthesized peptides to the endoplasmic reticulum (ER). A second signal, mediated by mannose-6-phosphate (M-6-P) modification of targeted proteins and M-6-P receptors (M-6-

Abbreviations: bp, base pair(s); kb, kilobase(s); RT-PCR, reverse transcription–polymerase chain reaction; cDNA, DNA complementary to RNA; M-6-P, mannose-6-phosphate; M-6-PR, M-6-P receptor; CD-M6PR, cation-dependent M-6-PR; ER, endoplasmic reticulum; I-cells, inclusion cells; ONPG, ortho-nitrophenyl-β-D-galactopyranoside; FCS, fetal calf serum; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescence protein; RFP, red fluorescence protein; GlcNAc-1-phosphotransferase, UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase; GlcNAc-1-phosphotransferase-γ, GlcNAc-1-phosphotransferase-γ subunit; CURL, compartment uncoupling receptor and ligand.

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PR) recognizing M-6-P signal, functions to sort soluble lysosomal proteins from ER to lysosomes, and therefore prevent them from being secreted out of cells (Kornfeld, 1987; Dahms et al., 1989). In this later process, specific modification of mannose residues linked to the targeted proteins plays an important role in the transportation of these molecules to lysosomes, although there are also M-6-PR-independent pathways responsible for the sorting of a part of the membrane-integrated and soluble lysosomal enzymes (Hunziker and Geuze, 1996; Rouille et al., 2000).

Biochemical studies have demonstrated that, in the M-6-P modification of lysosomal enzymes, UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) is responsible for transferring the GlcNAc-1 phosphate from UDP-GlcNAc to 1.6 and 1.3 branches of high mannose oligosaccharide chains on lysosomal enzymes to produce a phosphodiester. A second enzyme, *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase, catalyzes removing of GlcNAc from M-6-P, leaving the M-6-P residues linked to the oligosaccharide side chains of lysosomal enzymes, which are then recognized by M-6-PRs and sorted to lysosomes (Hasilik et al., 1980; Varki and Kornfield, 1980; Waheed et al., 1981; Couso et al., 1986; Kornfeld, 1987; Do et al., 2002). GlcNAc-1-phosphotransferase is regarded as a hexamer comprised of three types of subunits named α, β and γ (Bao et al., 1996a,b). Mutations of these proteins lead to failure to produce M-6-P epitope on soluble lysosomal enzymes, which will disrupt the trafficking of lysosomal enzymes and prevent them from entering lysosomes. These mutations have been identified as etiological causes of several types of mucolipidosis characterized by accumulation of macromolecules in cells, formation of large cytoplasmic vacuoles (I-cells) and intracellular loss of lysosomal enzymes (Neufeld, 1991; Owada, 1998). Further analyses of the type IIIC mucolipidosis suggested that the γ subunit of the GlcNAc-1-phosphotransferase (GlcNAc-1-phosphotransferase-γ) is responsible for substrate recognition but not catalysis activity of the enzyme (Varki et al., 1981; Lang et al., 1985; Raas-Rothschild et al., 2000). However, the exact molecular mechanism of the GlcNAc-1-phosphotransferase in catalyzing phosphate transfer is not yet fully understood.

In a screening of signal peptide-containing proteins from a mouse "hypothetical protein electronic library" constructed by searching protein databases of GenBank with the keyword: *Mus musculus* hypothetical protein, a molecule with serial number HYP36 was identified as the mouse homologue of the human GlcNAc-1-phosphotransferase-γ (Raas-Rothschild et al., 2000). Further analyses showed that overexpression of GlcNAc-1-phosphotransferase-γ in fibroblasts induced an I-cell-like phenotype that has been reported in cells from patients with mucolipidosis resulting from mutations affecting lysosomal enzyme-sorting pathway. These findings are helpful for the understanding of the mechanism of GlcNAc-1-phosphotransferase, and maybe informative for designing gene therapy of mucolipidosis type IIIC caused by mutations in GlcNAc-1-phosphotransferase.

2. Materials and methods

2.1. RT-PCR

Total RNA from mouse tissues and cell lines was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the procedure provided by the manufacturer. Reverse transcription was carried out using a kit from Invitrogen following the manufacturer's instruction. The first strand of cDNA was generated from 5 μg of total RNA by Superscript II reverse transcriptase using oligo-dT as a primer. The mouse GlcNAc-1-phosphotransferase-γ was amplified by PCR using primers: upstream primer, 5′-AAGAATTCGACCCTAGGAGCAATGGCG-3′, and downstream primer, 5′-AAAGATCTAGGATGTTCCCACGTAG-3′. The PCR products were fractionated by electrophoresis on 1% agarose gel and cloned into pMD18-T vector (Takara, Dalian, China) for sequencing.

For semi-quantitative examination of expression level of the mouse GlcNAc-1-phosphotransferase-γ, RT-PCR was performed using cDNA derived from different mouse tissues and cell lines. The mouse β-actin was amplified as an internal control, using primers 5′-GACGCGACCATCCTCCTCTTAG-3′ and 5′-TGCTTCTAGGCGGACTGTGTTACTGA-3′. The PCR products were analyzed by electrophoresis and the density of the bands was quantified using an image analyzer.

2.2. Northern blot

Total RNA (15 μg) from mouse liver and brain was size-fractionated by 1.2% formalin-denatured agarose gel electrophoresis, and transferred onto Hybond-N^+ membrane (Amersham Pharmacia Biotech). A cDNA probe specific for HYP36 (the mouse GlcNAc-1-phosphotransferase-γ) was generated by PCR, and ^{32}P-labeled using a random primer labeling kit (Promega). The membrane was hybridized with the probe, and HYP36 mRNA was visualized by autoradiography. Loading of total RNA was examined by stripping and reprobing the membrane using a human beta-actin probe.

2.3. Cell culture and transfection

COS7 and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS, Gibco-BRL), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate, in an atmosphere of 95% air–5% CO_2 at 37 °C. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's manual. Briefly, cells were plated in 6-well plates one day before transfection. After growing to