Molecular Genetics and Metabolism 109 (2013) 77–85

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

In vitro correction of disorders of lysosomal transport by microvesicles derived from baculovirus-infected *Spodoptera* cells

Jess Thoene [a,*], Thomas Goss [a], Marc Witcher [a], Jodi Mullet [a], Francisca N'Kuli [b], Patrick Van Der Smissen [b], Pierre Courtoy [b], Si Houn Hahn [c]

[a] Department of Pediatric Genetics, University of Michigan, Ann Arbor, MI, USA
[b] Cell Biology Unit, de Duve Institute, UCL Brussels, Belgium
[c] Department of Pediatrics, University of Washington, Seattle, WA, USA

ARTICLE INFO

Article history:
Received 7 January 2013
Received in revised form 24 January 2013
Accepted 24 January 2013
Available online 4 February 2013

Keywords:
Lysosomes
Transmembrane proteins
Cystinosis
Infantile sialic acid storage disease
Baculovirus

ABSTRACT

Infection of *Spodoptera frugiperda* (Sf9) cells by baculovirus (BV) is well established for transgene expression of soluble proteins, but few correctly folded transmembrane proteins have been so produced. We here report the use of the BV/Sf9 (BVES) method for the expression and transfer, via microvesicles, of the exclusive lysosomal exporters for cystine and sialic acid, human cystinosin and sialin. These proteins and their mRNA are released into the culture medium as very low-density microvesicles (~1.05 g/ml), which do not label for lysobisphosphatidic acid. The presence of the human transgene proteins in the vesicles was confirmed by western blotting and confirmed and quantified by mass spectrometry. Addition of vesicles to cultures of human fibroblast lines deficient in either cystinosin or sialin produced a progressive depletion of stored lysosomal cystine or sialic acid, respectively. The depletion effect was slow ($T_{1/2}$ ~48 h), saturable (down to ~40% of initial after 4 days) and stable (>one week). Surprisingly, BV infection of *Spodoptera* appeared to induce expression and release into microvesicles of the insect orthologue of cystinosin, but not of sialin. We conclude that BVES is an effective method to express and transfer functional transmembrane proteins so as to study their properties in mammalian cells, and has a generic potential for transport protein replacement therapy.

1. Introduction

Transmembrane proteins represent more than one-fourth of the human proteome and include plasma membrane receptors that account for the majority of current drug targets [1,2]. Their structural and functional study is hindered by the requirement to exist in a lipid bilayer, failure of which causes loss of function and formation of insoluble aggregates [3]. This drawback further precludes membrane protein replacement in deficient cells. Lysosomal enzyme replacement therapy is approved by the US FDA for 8 severe lysosomal substrate storage disorders characterized by hydrolase deficiencies: MPS I, II, IIIA, IV, Gaucher, Niemann–Pick, Fabry and Pompe diseases [4,5]. Disorders of lysosomal transport cause lethal human diseases due to storage of end degradation products [6], but replacement is not available due to difficulty in expressing and transferring functional transmembrane transporters. We here report the in vitro correction of two such disorders, Infantile Sialic Acid Storage Disease (ISSD) and cystinosis by engineered microvesicles.

ISSD is a rare autosomal recessive disorder which usually leads to neuronopathic death before an age of 2 years, and has no specific therapy [7,8]. ISSD is allelic with Salla disease, a less severe variant. Both forms result from mutations in SLC17A5, which encodes sialin, a lysosomal membrane transport protein for the acidic sugar, sialic acid. Sialin consists of 495 amino acids with twelve membrane-spanning regions (12TM). The pathophysiology of ISSD remains unclear [9]. In addition to lysosomal sialic acid storage, recent studies also report plasma membrane localization of sialin in primary neuron cultures [10], corroborating other studies that implicate sialin as a nitrate transporter at the plasma membrane, implying that NO balance may be perturbed by mutations in this gene [11].

Cystinosis is a rare autosomal recessive disease caused by mutations in CTNS, which codes for cystinosin, the sole lysosomal membrane exporter for the disulfide amino acid, cystine [12]. Despite a recent suggestion that cystinosin does not act directly as the cystine exporter, but as a chaperone for (an) other transporter(s) [13], functional characterization of cystinosin expressed in *Xenopus* oocytes demonstrated 1:1 proton/cystine symporter activity [14]. The major cystinosin isoform comprises 367 amino acids encompassing seven membrane-spanning domains (7TM) [15]. The most frequent mutation found in patients of Caucasian descent is a large 57 kb deletion which ablates protein

Abbreviations: BV, Baculovirus, also used as emptyBac (no transgene insert); sialBac, (BV containing the human sialin sequence); cysBac, (BV containing the human CTNS sequence); BVES, Baculovirus Expression System; Sf9, Spodptera frugiperda cell line 9 (from ovarian tissue).

* Corresponding author at: Biochemical Genetics Laboratory, Division of Pediatric Genetics, University of Michigan School of Medicine, 3725 Med Sci II, 1150 West Medical Center Dr, Ann Arbor, MI 48109-5629, USA.
E-mail address: jthoene@umich.edu (J. Thoene).

1096-7192/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymgme.2013.01.014

expression, but many point mutations have also been identified. Defective cystinosin leads to lysosomal cystine storage, and in the nephropathic form, produces profound failure to thrive and multisystemic lesions, the most severe being renal tubule dysfunction (renal Fanconi syndrome) followed by kidney failure by age ~10 years [16], possibly due to enhanced apoptosis [17,18]. Cystinosis is currently treated with 2-aminoethanethiol (Cystagon) [19], which removes stored lysosomal cystine via a mixed disulfide reaction, the product of which is exported via the intact lysine transporter [20,21]. This treatment delays kidney failure by about 10 years, but does not provide a cure, and causes significant compliance issues due to the taste and smell of the free thiol.

Baculovirus (BV) is a natural pathogen for Lepidoptera, where virion entry leads to a lytic infection causing death of the organism. The baculovirus transgene expression system in cultured *Spodoptera* cells has been used for over 25 years to express >1000 recombinant genes [22]. Although a few membrane receptor proteins have been expressed and characterized in this system, including the 7TM beta-adrenergic receptor [23,24], functional lysosomal membrane transport proteins have not been successfully produced via BVES. In this paper we report its first use to produce functional human cystinosin and sialin. We find that upon infection of Sf9 cells with transgene-bearing BV, a heterogeneous population of microvesicles is released into the conditioned medium that contains both the transgene message and its translational product. Moreover, these microvesicles specifically decrease the stored lysosomal material when added to mutant human fibroblast cultures, a correction that is only attributable to the expressed protein. Surprisingly, BV infection of Sf9 cells by itself resulted in the induction and release in vesicles of a putative insect orthologue of cystinosin, but not of sialin, for which no Lepidopteran orthologue is known.

2. Experimental procedures

2.1. Cell culture

Normal and mutant human fibroblast lines were purchased from the Corriell Institute (Camden NJ), and cultured in Ham's F12 media with 15% FCS and Pen/Strep under 5% CO_2/95% air [17]. Sf9 cells (Invitrogen, Carlsbad, CA) were cultured in SF 900 II insect media according to supplier's instructions. Cell protein was measured by the bicinchoninic acid method [25] using BSA as standard.

2.2. Assay of sialic acid and cystine

ISSD cells (GM 09885) were harvested by trypsinization and the cell pellet frozen in 150 μl of distilled water and shipped to Dr D. A. Wenger at Jefferson Medical College, Department of Neurology (Philadelphia, PA) for measurement of free and protein-bound sialic acid. *Free sialic acid* was quantified after periodate oxidation to malonaldehyde and reaction with thiobarbituric acid, and read at 549 nm [26]. Results are expressed as nmol/mg cell protein. *Total sialic* acid is the same procedure after acid hydrolysis, and bound is the difference between total and free. *Free cystine.* Fibroblasts were harvested by trypsinization, acid-precipitated with 12% sulfosalicylic acid, and the soluble supernatant sent for cystine analysis by tandem mass spectrometry at the Cystine Determination Laboratory (UCSD, CA). Results are expressed as nmol cystine/mg cell protein.

2.3. Conditioned media from BV-infected Sf9 cells

Conditioned media from BV-infected Sf9 cells was prepared by infection of cultured Sf9 cells in SF 900 II serum free insect culture media with BV containing the transgene of interest and incubated for 4 days at 27 °C at an MOI of 1.0. Conditioned media was prepared at the University of Michigan, and also purchased from the University of Iowa Gene Transfer Vector Core, and from Genscript (Piscataway, NJ 08854, USA). BV with the His-tagged, insect optimized CTNS and SLC17A5 sequences were obtained from Genscript, using their proprietary method of optimization accessible at http://www.genscript.com/codon_opt.html.

2.4. Microvesicle isolation

Since the insect culture media (SF900-II) used to grow Sf9 cells are toxic to human fibroblasts, derived microvesicles were transferred to mammalian tissue culture media for depletion studies. This was accomplished by either 1) passing the conditioned media through a 0.2 μm filter followed by dialysis (cut-off 3.5 kDa) from insect culture medium into mammalian tissue culture medium at a final effective dilution of 1/2500, or 2) by ultracentrifugation (140,000$\underline{g}\times$3 h), followed by resuspension in Ham's F12 with fetal calf serum and antibiotics to the initial volume. In all experiments, microvesicles were again filter-sterilized prior to addition to fibroblast cultures. For consistency, microvesicles obtained from 24 ml of conditioned media were equally divided among 2×100 mm semi-confluent plates of ISSD fibroblasts (or from 12 ml into three 60 mm plates of cystinotic cells). The plate size difference was dictated by the lower assay sensitivity for sialic acid than for cystine, and because two samples are required for separate analysis of free and bound sialic acid.

2.5. Subcellular fractionation of microvesicles

The entire procedure was carried out under sterile conditions. Frozen conditioned medium was shipped to Brussels. Differential centrifugation allowed (i) elimination of cell debris and large material (18,000$\underline{g}\times$min); (ii) removal of large particles from the first supernatant (Beckman Ti50; ~400,000$\underline{g}\times$min); and (iii) pelleting microvesicles from the second supernatant (Ti50; ~60 $10^6\underline{g}\times$min). This high-speed pellet was gently resuspended in 250 μL of 0.25 M sucrose/3 mM imidazole/1 mM EDTA and further resolved by isopycnic floatation (mixing with 750 μL 60% Optiprep, loading under preformed linear 40%–6% Optiprep gradient and centrifugation at 90×$10^6\underline{g}\times$min). Eight 1-mL fractions were collected from the top by reference to a parallel tube with rainbow density bead markers (Amersham) and analyzed for density (from weight), protein (SDS-PAGE at 12% acrylamide and silver staining), and LBPA (dot blot: #6CA mouse monoclonal 1/200 from Tebu-Bio; revealed by HRP-goat anti-mouse IgG from Biosource and enhanced chemiluminescence). Results were expressed as percentage of the sum of all fractions analyzed in the same film. Each fraction was pelleted again at 90×$10^6\underline{g}\times$min and re-suspended in culture medium to assess for cystine depletion bioactivity in cystinotic GM0008 fibroblasts.

2.6. Scanning electron microscopy of fibroblasts

After 96 h incubation with vesicles, cells were immediately fixed in 3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Sorensen's buffer. They were then washed in Sorenson's buffer with two additional changes of buffer before osmium fixation and imaging.

2.7. Qualitative identification of human sialin and cystinosin

Qualitative identification of human sialin and cystinosin was performed by the University of Michigan Proteomics and Peptide Synthesis Core. Vesicles were disrupted in 200 μL of modified RIPA using a Bullet Blender and the extracted protein was quantitated by Qubit Fluorometry (Invitrogen). 20 μg of material was separated on a 4–12% Bis Tris NuPage gel (Invitrogen) in MOPS buffer and divided into twenty equally sized segments. Gel pieces were processed using a robot (ProGest, DigiLab) with the following protocol: (i) wash with 25 mM ammonium bicarbonate followed by acetonitrile; (ii) reduction by 10 mM dithiothreitol at 60 °C followed by alkylation with 50 mM iodoacetamide; (iii) digestion with trypsin (200 ng/gel band, Promega) at 37 °C for 4 h; and (iv) quenching with formic acid. The final