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Common traffic routes for imported spermine and endosomal glypican-1-derived heparan sulfate in fibroblasts

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ABSTRACT

Import of the polyamine spermine from the extracellular environment depends on the presence of cell surface heparan sulfate proteoglycans, such as glypican-1. This proteoglycan is internalized by endocytosis, releases its heparan sulfate chains in endosomes by a nitric oxide-, copper- and amyloid precursor protein-dependent mechanism, then penetrates the membrane and is transported to the nucleus and then to autophagosomes. This process is spontaneous or induced by ascorbate depending on the growth-state of the cell. Here, we have explored possible connections between the heparan sulfate traffic route and spermine uptake and delivery in wild-type and Tg2576 mouse fibroblasts. Cells were examined by deconvolution immunofluorescence microscopy. The antibodies used were specific for spermine, glypican-1-derived heparan sulfate, Rab7, nucleolin and a marker for autophagosomes. Endogenous immunostainable spermine was primarily associated with autophagosomes. When spermine synthesis was inhibited, imported spermine appeared in Rab7-positive endosomes. When ascorbate was added, heparan sulfate and spermine were transported to the nucleus where they colocalized with nucleolin. Spermine also appeared in autophagosomes. In a pulse-chase experiment, heparan sulfate and spermine were first arrested in late endosomes by actinomycin D treatment. During the chase, when arrest was abolished, heparan sulfate and spermine were both transported to the nucleus and targeted nucleolin. In amyloid precursor protein$^{-/-}$-fibroblasts, ascorbate failed to induce release of heparan sulfate and spermine remained in the endosomes. We propose that cell surface glypican-1 carries spermine to the endosomes and that the released heparan sulfate carries spermine across the membrane into the cytosol and then to the nucleus.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are polycationic compounds that are essential for a wide variety of cellular functions including proliferation, differentiation and survival. The polyamine levels in cells are regulated by biosynthesis, degradation and import from the extracellular environment. Intracellular polyamines bind electrostatically to polyanionic macromolecules, such as DNA and RNA. The majority of cellular polyamines are bound to RNA (for reviews, see [1,2]). Sulfated glycosaminoglycans, such as dermatan sulfate and heparan sulfate (HS), which are polyanionic components of extracellular as well as cell-surface proteoglycans, bind spermine (SPM) with affinities sometimes greater than that for binding to DNA [3,4].

When ornithine decarboxylase, the key enzyme in polyamine biosynthesis, is inhibited by α-difluoromethylornithine (DFMO), cells become dependent on uptake of polyamines from the environment. In fibroblasts, there is an almost ten-fold increase in SPM uptake. Addition of HS or enzymatic removal of cell surface HS markedly reduces uptake [5]. Since then, numerous reports have shown that efficient uptake of basic compounds, including SPM, depends on the presence of cell surface-associated HS proteoglycans, such as the syndecans and glypicans [6].

Glypican-1 (Gpc-1) can be internalized by endocytosis, followed by release of the HS chains by nitric oxide (NO)-dependent deaminative cleavage in endosomes, and recycled via the Golgi [7,8]. Deaminative cleavage can be spontaneous or induced by exogenously supplied ascorbate [9]. Uptake of SPM by human bladder carcinoma T24 cells was studied earlier in this laboratory by using a monoclonal antibody against SPM (mAb Spm8-2). T24 cells were first transfected with unspecific or Gpc-1-specific siRNA. After treatment with DFMO followed by incubation with SPM, cells were treated with ascorbate to release HS from the Gpc-1 core protein. Subsequent confocal immunofluorescence

Abbreviations: Aβ, amyloid beta peptides; anMan, anhydromannose; APP, amyloid precursor protein; DAPI, 4,6-diaminido-2-phenylindole; DFMO, α-difluoromethylornithine; DMEM, Dulbecco minimal essential medium; Gpc-1, glypican-1; HS, heparan sulfate; MEF, mouse embryonic fibroblasts; MEM, minimal essential medium; NCL, nucleolin; NO, nitric oxide; SNO, S-nitrosothiol; SPM, spermine; Tg2576, Alzheimer transgene; WT, wild-type

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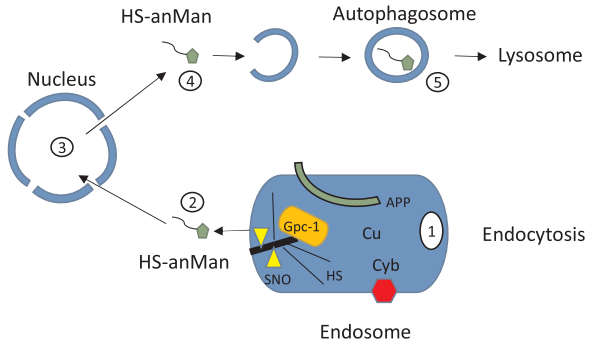

Fig. 1. A schematic presentation of the proposed traffic route for glypican-1 (Gpc-1) processing and recycling. 1, Endocytosed Gpc-1 is S-nitrosylated (SNO) in a Cu-dependent reaction supported by the amyloid precursor protein (APP). Ascorbate-induced release of anhydromannose-containing heparan sulfate (HS-anMan) from Gpc-1-SNO in endosomes requires both cytochrome b561 (Cyb), Cu and β-cleavage of APP; 2, HS-anMan penetrates the membrane; 3, HS-anMan enters the nucleus; 4, HS-anMan leaves the nucleus; 5, HS-anMan is captured in autophagosomes and eventually degraded in lysosomes.

microscopy showed that substantial amounts of SPM had been sequestered by intracellular HS in cells expressing Gpc-1, while suppression of Gpc-1 expression had effectively prevented uptake. SPM taken up by the control cells was found in cytoplasmic compartments as well as in the nuclei [10].

Recently, we observed that when wild-type mouse embryonic fibroblasts (WT-MEFs) were incubated with ascorbate for short periods of time (5–30 min), HS was released from Gpc-1 in late endosomes, penetrated the membrane and was transported to the nucleus. HS then disappeared from the nucleus and was captured in autophagosomes and degraded in lysosomes [11]. A schematic presentation of the proposed traffic route is shown in Fig. 1. Ascorbate-induced release of HS from Gpc-1 in endosomes involves cytochrome b561, copper ions, and beta-cleavage of the Alzheimer amyloid precursor protein (APP) and results in the formation of anhydromannose (anMan)-containing HS [11,12]. Nuclear HS-anMan colocalizes with nucleolin (NCL) suggesting that NCL carries HS-anMan to the nucleus and that nucleoli are primary targets [13]. We therefore decided to explore possible connections between the HS traffic route and SPM uptake and delivery to various subcellular sites.

2. Materials and methods

2.1. Materials

MEF cells from wild-type (WT), APP$^{-/-}$ and the Alzheimer Tg2576 mice were the same as described earlier [11–13]. Minimal essential medium (MEM) was used as regular growth medium and Iscove-Dulbecco minimal essential medium (DMEM) for spermine uptake. Spermine (SPM), the DNA staining compound 4,6-diaminido-2-phenylindole (DAPI) and ascorbic acid were obtained from Sigma-Aldrich, α-difluoromethylornithine (DFMO) was from ILEX Oncology and Actinomycin D from Fisher scientific. [^{14}C]-SPM hydrochloride (50 μCi/ml) was obtained from Amersham. The monoclonal antibody against HS-anMan (mAb AM) was originally described by Pejler et al. [14]. The monoclonal antibody against SPM (mAb spm8-2) was the same as used previously [10]. The polyclonal antibodies were: anti-nucleolin (NCL, ab22578, Abcam), anti-LC3 (L8918, Sigma) and anti-Rab7 (sc10767, Santa Cruz). FITC-labeled goat anti-mouse IgG was purchased from Sigma and Alexa-Fluor 594-labeled goat or donkey anti-rabbit IgG from Life Technologies.

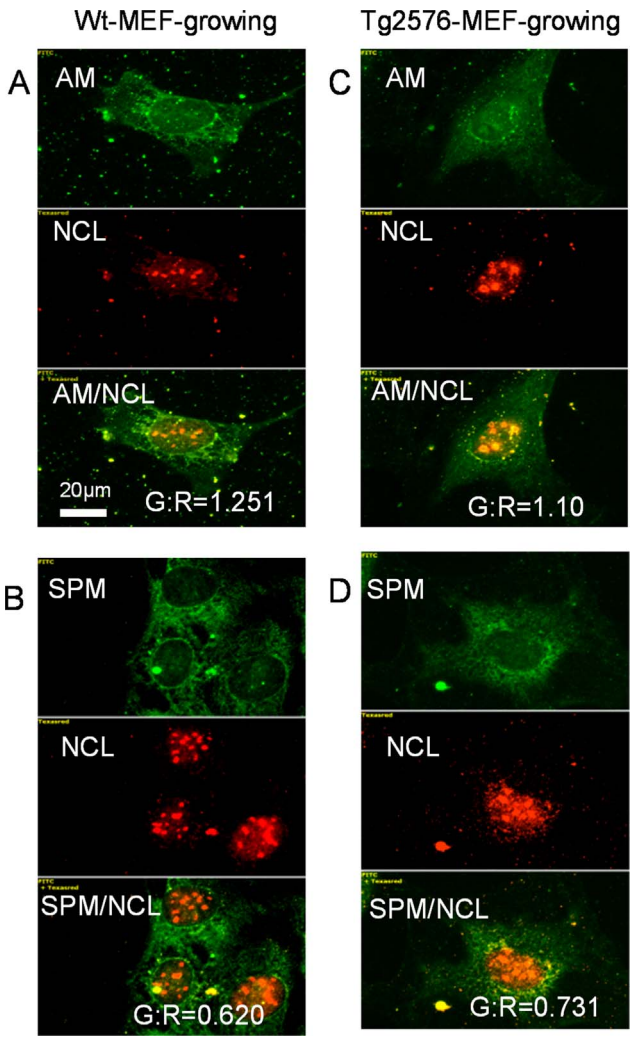

Fig. 2. HS-anMan and SPM are found both in the nuclei and the cytoplasm of growing fibroblasts. Representative immunofluorescence images of growing WT-MEF cells (A-B) and Tg2576-MEF cells (C-D). Staining was performed with mAbs anti-HS-anMan (AM) and spm8-2 (SPM), and a polyclonal antibody against nucleolin (NCL). Exposure time was the same in all cases. The extent of colocalization is expressed as the G:R ratio (green versus red channel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Deconvolution immunofluorescence microscopy

Cells were examined by immunofluorescence microscopy as described previously [11]. In brief, cells were fixed in acetone in order to retain cellular and subcellular structures and to ensure the preservation of carbohydrates. The fixed cells were first pre-coated with 10% anti-mouse total Ig and then exposed to primary antibodies overnight. The secondary antibodies used were FITC-tagged goat antimouse Ig when the primary antibody was a monoclonal and Alexa Fluor 594-tagged goat anti-rabbit IgG or sometimes Alexa Fluor 594-tagged donkey anti-rabbit IgG when the primary antibody was a polyclonal. In the controls, the primary antibody was omitted. DNA staining with 4,6-diamidino-2-phenylindole (DAPI), as