ScFv Antibody-induced Translocation of Cell-surface Heparan Sulfate Proteoglycan to Endocytic Vesicles

EVIDENCE FOR HEPARAN SULFATE EPITOPE SPECIFICITY AND ROLE OF BOTH SYNDECAN AND GLYPICAN

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Anders Wittrup 1, Si-He Zhang 2, Gerdy B. ten Dam 3, Toin H. van Kuppevelt 4, Per Bengtson 1, Maria Johansson 1, Johanna Welch 5, Matthias Mørgelin 6, and Mattias Belting 1,2

From the 1Section of Oncology and 5Section of Clinical and Experimental Infectious Medicine, Department of Clinical Sciences, Lund University, SE-221 85 Lund, Sweden and the 5Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, 6500 HB Nijmegen, The Netherlands

Cellular uptake of several viruses and polybasic macromolecules requires the expression of cell-surface heparan sulfate proteoglycan (HSPG) through as yet ill defined mechanisms. We unexpectedly found that among several cell-surface-binding single chain variable fragment (scFv) anti-HS antibody (αHS) clones, only one, AO4B08, efficiently translocated macromolecular cargo to intracellular vesicles through induction of HSPG endocytosis. Interestingly, AO4B08-induced PG internalization was strictly dependent on HS 2-O-sulfation and appeared independent of intact N-sulfation. AO4B08 and human immunodeficiency virus (HIV)-Tat, i.e. a well known cell-penetrating peptide, were shown to compete for the internalizing PG population. To obtain a more detailed characterization of this pathway, we have developed a procedure for the isolation of endocytic vesicles by conjugating AO4B08 with superparamagnetic nanoparticles. [35S]sulfate-labeled HSPG was found to accumulate in isolated, AO4B08-containing vesicles, providing the first biochemical evidence for intact HSPG co-internalization with its ligand. Further analysis revealed the existence of both syndecan, i.e. a transmembrane HSPG, and glycosyl-phosphatidylinositol-anchored glypicin in purified vesicles. Importantly, internalized syndecan and glypicin were found to co-localize in AO4B08-containing vesicles. Our data establish HSPGs as true internalizing receptors of macromolecular cargo and indicate that the sorting of cell-surface HSPG to endocytic vesicles is determined by a specific HS epitope that can be carried by both syndecan and glypican core protein.

Endocytosis regulates many processes, including signaling events involved in cell motility and cell fate determination, nutrient uptake, microbial invasion, and macromolecular drug delivery. Cellular internalization of highly diverse macromolecular ligands, including polylysine/cationic lipid-DNA complexes, cationic polymers, antimicrobial peptide-DNA complexes, and “naked” DNA, have been shown to depend on cell-surface heparan sulfate proteoglycans (HSPGs) that are expressed on virtually all mammalian cells (1–6). Together with the fact that several viruses utilize HSPGs for cell-surface adsorption and internalization (7, 8), HSPG emerges as an important target molecule that should be considered in the development of drug delivery vehicles.

HSPGs are a class of proteins substituted with glucosamine-glucuronic acid disaccharide polysaccharides (9, 10) that are extensively modified during/subsequent to polymerization, including N-deacetylation/sulfation and 6- and 3-O-sulfation of the glucosamine, epimerization at C-5 of glucuronic acid into iduronic acid, and 2-O-sulfation (2-OS) of iduronic acid. These modifications provide the HS chains with complex patterns of sulfation and high negative charge that largely determine their functional interactions with polybasic ligands (8, 9, 11, 12). There are two major classes of HS-bearing cell-surface PGs: six members of the glypicin (GPC) family of glycosyl-phosphatidylinositol (GPI)-linked proteins (13, 14) and four members of the syndecan (SDC) family of transmembrane proteins (15, 16). Due to their differential modes of membrane association, GPC and SDC are generally believed to present HS chains to different plasma membrane microdomains to exert specific biological functions. Although previous reports have documented an important role of HSPG in cellular internalization of numerous macromolecules (8), some key questions on the exact function of HSPG in this process have remained unanswered, i.e. few studies have focused on the fate of cell-surface HSPG and the specific roles of SDC and GPC during macromolecular delivery. Instead, the influence of HS ligand properties, e.g. peptide interactions with their ligand. Further analysis revealed the existence of both syndecan, i.e. a transmembrane HSPG, and glycosyl-phosphatidylinositol-anchored glypicin in purified vesicles. Importantly, internalized syndecan and glypicin were found to co-localize in AO4B08-containing vesicles. Our data establish HSPGs as true internalizing receptors of macromolecular cargo and indicate that the sorting of cell-surface HSPG to endocytic vesicles is determined by a specific HS epitope that can be carried by both syndecan and glypican core protein.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4 and supplemental Table 1.
3 To whom correspondence may be addressed: Dept. of Clinical Sciences, Section of Oncology, Lund University, Barnsgatan 2:1, SE-221 85 Lund, Sweden. Tel.: 46-46-178549; E-mail: anders.wittrup@med.lu.se.
4 To whom correspondence may be addressed: Dept. of Clinical Sciences, Section of Oncology, Lund University, Barnsgatan 2:1, SE-221 85 Lund, Sweden. Tel.: 46-46-178549; E-mail: mattias.belting@med.lu.se.
5 The abbreviations used are: HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PG, proteoglycan; αHS, scFv anti-HS antibody; HIV, human immunodeficiency virus; scFv, single chain variable fragment; αHS-F, fluorescent αHS complex; BSA, bovine serum albumin; CHQ, Chinese hamster ovary; GFP, glypicin; GPC, glycosyl-phosphatidylinositol; NS, N-sulfation; PNS, postnuclear supernatant; SDC, syndecan; 2-OS, 2-O-sulfation; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HA, hemagglutinin.
sequence, length, and net positive charge on the intracellular fate of delivered macromolecules, has been extensively studied (17, 18). Indeed, the role of HS proteoglycans (HSPGs) in the uptake of macromolecular cargoes remains controversial. They have been proposed to act either as true internalizing receptors or as receptors for initial cell-surface attachment. In the latter case, ligand binding and concentration by the HSPG would result in subsequent ligand presentation to alternative, internalizing receptors or in ligand uptake through receptor-independent pinocytosis or even in direct membrane penetration through phospholipid interactions (5, 8, 18, 19).

van Kuppevelt and co-workers (20) have previously described the development and characterization of epitope-specific, phage display-derived, scFv anti-HS antibodies (αHS) that can be utilized to probe the structural diversity of HS chains in various tissues and cell types. Here, using several of these αHS in conjunction with the development of a strategy for the isolation of endocytic vesicles, we address the internalizing function of cell-surface HSPG and investigate the respective roles of HS ligand epitope specificity and PG core protein specificity in macromolecular delivery.

**EXPERIMENTAL PROCEDURES**

**Materials**

Na$_2^{35}$SO$_4$ (1310 Ci/mmoll) was from PerkinElmer Life Sciences, HIV-Tat peptide (GRKKRRQRRRRPPQC) was from Innovagen AB, Lund, Sweden, and YOYO-1 and fluorophore-labeled antibodies were from Invitrogen. MagCellect magnetic nanoparticle-conjugated goat anti-mouse IgG was from R&D Systems. N-terminally tagged full-length rat HA-GPC3 (21) constructs were kindly provided by Dr J. Filmus, University of Toronto, Canada. Full-length rat SDC2 and SDC3 cloned into pEGFP-N3 vector with a C-terminal GFP tag and intact sorting and glycosylation (22)$^4$ was a kind gift from Dr. P. Landgraf, Leibniz-Institute for Neurobiology, Magdeburg, Germany. αHS antibodies were obtained by biopanning against HS isolated from bovine kidney (HS4E4, HS4C3), from skeletal muscle from mouse (AO4B08) and human (RB4EA12), and from human lung (EV3C3) as described (23, 24). Chondroitinase ABC lyase, heparanases I and III, mouse monoclonal anti-vsv (P5D4), mouse monoclonal anti-myc (9E10), and rabbit polyclonal anti-vsv antibodies, cell media and supplements, and fine grade chemicals were from Sigma.

**Cell Culture**

Wild-type Chinese hamster ovary (CHO)-K1 cells, PG-deficient pgsA-745, HSPG-deficient pgsd-677 CHO cell mutants (25), human cervix adenocarcinoma (HeLa), and lung carcinoma (A549) cells were from the American Type Culture Collection (ATCC). N-sulfation (NS)-deficient pgsF-606 (26) and 2-OS-deficient pgsF-17 (27) CHO cells were kindly provided by Dr J. D. Esko, University of California, San Diego. CHO and A549 cells were routinely cultured in F12K, and HeLa cells were cultured in DMEM in a humidified 5% CO$_2$ incubator at 37 °C, using the respective medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Flow Cytometry**

For antibody binding, cells were detached with PBS (2×)/0.5 mM EDTA, washed with PBS BSA (1% w/v), and incubated with αHS (titer 1:20) and then washed in PBS BSA and incubated with mouse anti-vsv antibody (1:500) that recognizes the vsv epitope of αHS antibodies followed by rinsing in PBS BSA and incubation with goat anti-mouse-Alexa Fluor 488 antibody (1:200). All antibody incubations were performed in PBS BSA for 30 min on ice. Finally, cells were washed in PBS BSA and analyzed by flow cytometry on a FACSCalibur instrument integrated with CellQuest software (BD Biosciences). For uptake experiments, αHS (1:20), mouse anti-vsv (1:500), and goat anti-mouse-Alexa Fluor 488 antibodies (1:200) were precomplexed in serum-free medium at 20 °C for 30 min (αHS-F) and then incubated with cells at 37 °C for 1 h, unless otherwise stated. Cells were then trypsinized, suspended in DMEM 10% fetal bovine serum, washed in PBS BSA, and analyzed by flow cytometry. Controls without αHS primary antibody were included in all binding and uptake experiments.

**Cell-surface HS Epitope Consumption Assay**—Cells were incubated either with Tat (25 μg/ml), DNA/Tat (10/40 μg/ml), precomplexed AO4B08 (1:40), and mouse anti-vsv antibody (1:500) or with precomplexed myc-tagged AO4B08 and anti-myc secondary antibody (1:100) for various periods of time at 37 °C. Control cells were incubated with the above ligands for 30 min at 4 °C. Cells were then washed twice with PBS 1% NaCl, to remove surface-bound ligands, and detached with PBS (2×)/0.5 mM EDTA followed by surface staining with AO4B08 or RB4EA12 as described above under antibody binding.

**PG Core Protein Overexpression**—HeLa cells were transfected with GFP, SDC2-GFP, SDC3-GFP, or both GFP and HA-GPC3, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and cultured for another 36–48 h to allow for plasmid gene expression. AO4B08-F (Alexa Fluor 633) uptake for 1 h was determined in non-transfected and transfected cells by flow cytometry.

**PG Core Protein Knockdown**—HeLa cells were transfected with predesigned small interfering RNA (Ambion) with Lipofectamine 2000 according to the manufacturer’s instructions.

**Confocal Fluorescence Microscopy**

HeLa cells grown in chamber slides were incubated with AO4B08-F at 37 °C for various periods of time. Cells were then rinsed with PBS 1% NaCl to remove surface-associated ligand and fixed in 4% (w/v) paraformaldehyde. ToPro (1:100) nuclear stain was performed after permeabilization with 0.1% Triton X-100. In another series of experiments, HeLa cells transfected with SDC2-GFP, SDC3-GFP, HA-GPC3, or both SDC2-GFP and HA-GPC3 were incubated with AO4B08 (1:40) and rabbit anti-vsv (1:500) antibody complexes for 10–30 min at 37 °C, washed twice with PBS 1% NaCl, fixed and permeabilized, blocked for 30 min in 2% BSA, and stained with goat anti-rabbit-Alexa Fluor 546 (1:500) (SDC2-GFP- and SDC3-GFP-transfected) or with mouse anti-HA (1:200) followed by goat anti-mouse-Alexa Fluor 488 (1:500) and goat anti-rabbit-Alexa

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$^4$ P. Landgraf, personal communication.
Fluor 546 (1:500) antibodies (HA-GPC3-transfected) or with mouse anti-HA (1:200) followed by goat anti-mouse-Alexa Fluor 546 (1:500) and goat anti-rabbit-Alexa Fluor 647 (1:500) antibodies (SDC2-GFP- and HA-GPC3-transfected). For co-incubation experiments, cells were incubated with AO4B08-myce (1:40) and mouse anti-myce (1:100) complexes together with RB4EA12-vsv (1:40) and rabbit anti-vsv (1:500) antibody complexes for 30 min at 37 °C and stained with goat anti-mouse-Alexa Fluor 546 (1:500) and goat anti-rabbit-Alexa Fluor 488 (1:500) after permeabilization. Cells were mounted in PermaFluor (Beckman Coulter) and analyzed using Zeiss LSM710 confocal scanning equipment with a 63 × 1.4 NA oil immersion objective.

**Magnetic Vesicle Purification**

AO4B08 (titer 1:40), mouse anti-vsv (1:500), and magnetic MagCellect goat anti-mouse (1:20) antibodies were allowed to form complexes (AO4B08-M) in DMEM at 20 °C for 30 min. AO4B08-M was then added to HeLa cells at 37 °C for various periods of time followed by washing with PBS, trypsin detachment, suspension in DMEM 10% fetal bovine serum, and washing with PBS. Complete protease inhibitor (Roche Applied Science) was included in all subsequent steps. Cells were then mechanically disrupted by an optimized procedure, including passage through a 27-gauge needle during 25 strokes, the addition of CaCl2 (final concentration, 6 mM) and Benzonase (Sigma), and incubation for 20 min at 4 °C. Remaining intact cells, cell debris, and nuclei were removed by centrifugation at 500 × g for 5 min. The resulting supernatant was pooled with the supernatant obtained after a second centrifugation at 300 × g for 3 min to yield a postnuclear supernatant (PNS), which was separated into a magnetic and non-magnetic fraction in a magnetic separator (PickPen, Bio-Nobile). Protein amounts were quantified using an EZQ kit (Invitrogen). To evaluate the purification efficiency, non-magnetic and magnetic fractions of the PNS from cells incubated with AO4B08-F and AO4B08-M were analyzed using a Zeiss Axiovert fluorescence microscope with a 25 × 0.8 NA oil immersion objective, or using a FACSAria cell sorter (BD Biosciences) with the ND FSC filter removed to allow detection of small particles.

**Electron Microscopy**

HeLa cells incubated with AO4B08-M at 37 °C for various periods of time were washed once with PBS, detached by trypsin, suspended in DMEM 10% fetal bovine serum, washed twice in Tris-buffered saline, pelleted, and fixed for 1 h at 20 °C and overnight at 4 °C in 2.5% glutaraldehyde in cacodylate buffer. Cells were washed with cacodylate buffer, postfixed for 1 h at 20 °C in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded series of ethanol, and then embedded in Epon 812 using acetone as an intermediate solvent. Specimens were cut into 50-nm sections on an LKB Instruments ultramicrotome and stained with uranyl acetate and lead citrate. Alternatively, isolated membrane vesicles were prepared and analyzed as above or by negative staining and electron microscopy as described previously (28). Specimens were observed in a Jeol JEM 1230 electron microscope operated at 60-kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera.

**Isolation and Analysis of HSPG**

Subconfluent HeLa cells were prelabeled with Na235SO4 (100 µCi/ml) in sulfate-deficient medium for 24 h. Cell layers were then extensively washed with PBS and incubated with AO4B08-M at 37 °C for various periods of time, and the PNS of disrupted cells was fractionated into non-magnetic and magnetic fractions as described above. Triton X-100 was added (final concentration 2%), and samples were solubilized for 20 min at 20 °C. [35S]PGs were then purified using DEAE-cellulose anion exchange chromatography and further analyzed by Superose 12 HR gel chromatography and scintillation counting or by gel electrophoresis in a 4–12% NuPAGE (Invitrogen) as described previously (29). In some cases, isolated [35S]PGs were treated with chondroitinase ABC and/or heparanase I/III digestion, as described (29), prior to electrophoresis. [35S]PGs were visualized using Fujifilm BAS-5000 equipment and quantified by densitometry using TotalLab v1.11 software.

For immunoblotting, separated proteins were transferred to polyvinylidene difluoride membrane (Pall Corp.), incubated with mouse monoclonal anti-DHS (3G10, Seikagaku, Japan) (1:1000) or rabbit polyclonal anti-GPC1 (kindly provided by Dr. K. Mani, Lund University) (1:1000), goat polyclonal anti-SDC2 (L-18, Santa Cruz Biotechnology) (1:200), or mouse monoclonal anti-SDC1 (B-B4, Serotec, a kind gift from Dr. A. Malmström, Lund University) (1:50) antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Protein bands were visualized using ECL Western blotting substrate (Pierce) and x-ray film (Hyperfilm MP, Amersham Biosciences).

For real-time quantitative PCR, cellular RNA was extracted using GenElute mammalian total RNA kit (Sigma), and cDNA synthesis was done using Superscript III Platinum two-step qRT-PCR kit (Invitrogen). Diluted cDNA was used for quantitative PCR based on SYBR Green I chemistry (Sigma) in an ABI PRISM 7900 HT machine (Applied Biosystems). Relative quantification of PG core protein expression as compared with β-actin was performed on triplicates of each reaction.

**Statistical Analyses**

Microscopy, flow cytometry, and gel electrophoresis experiments are representative of at least three independent experiments. Data points in flow cytometry experiments in Figs. 1 and 2 are the mean ± S.D. (n = 3). In some cases (Figs. 1 and 2), the error bars were smaller than the drawn symbols.

**RESULTS**

**Binding and Uptake of αHS—**A collection of αHS with the HS epitope specificities as indicated in supplemental Table 1 were screened for surface binding and uptake in HeLa cells. To investigate the potential of αHS to deliver a macromolecular cargo, we took advantage of their specific vsv tag to form non-covalent complexes with fluorophore-conjugated antibodies (αHS-F; see “Experimental Procedures”). Several of the tested αHS clones were shown to bind to the surface of HeLa cells. Interestingly, only one of the antibodies, AO4B08, was effi-
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ciently internalized (Fig. 1A). AO4B08-F and RB4EA12-F were further analyzed for binding and uptake in A549 cells (Fig. 1B). Again, AO4B08-F and RB4EA12-F showed comparable binding, but only AO4B08-F was internalized, indicating that specific internalization of AO4B08-F was not restricted to HeLa cells.

AO4B08-F uptake displayed saturable kinetics and time dependence with a linear increase over at least 3 h of incubation (supplemental Fig. 1, A and B). Insignificant uptake at 4 °C (data not shown) further suggested endocytic uptake, which was supported by confocal microscopy studies (Fig. 1C and supplemental Fig. 1C) as well as by electron microscopy studies of cells incubated with AO4B08 as a complex with magnetic nanoparticle-conjugated antibody (Fig. 1D). Importantly, AO4B08-F uptake was strictly dependent on HS expression, as evidenced by negligible uptake in HS lyase-treated HeLa cells, as well as in pan PG-deficient (pgsA-745) and HSPG-deficient (pgsD-677) CHO cell mutants (Fig. 1E). Moreover, HeLa cells treated with chondroitinase ABC, which degrades chondroitin/dermatan sulfate, showed unperturbed uptake of AO4B08-F (Fig. 1E). Strikingly, AO4B08-F uptake was totally abolished in 2-OS-deficient cells (pgsF-17), whereas uptake was unperturbed in NS-deficient cells (pgsE-606) (Fig. 1F). RB4EA12-F uptake was almost insignificant in wild-type as well as in sulfation-deficient mutant cells.

HIV-Tat and AO4B08 Compete for Internalizing. Cell-surface HSPG—Previous studies have shown that HIV-Tat delivers various macromolecular cargoes through an HSPG-dependent pathway (3). However, it remains ill-defined whether HSPG, irrespective of the mechanism of ligand uptake, acts as an attachment site or is co-internalized with its cargo. Cell-surface binding (Fig. 2A) and uptake (Fig. 2B) of HIV-Tat were inhibited by AO4B08 in a dose-dependent manner. Importantly, this was true for both AO4B08 monomers and AO4B08 antibody complexes (data not shown), i.e. AO4B08 multimerization was not required for competing off HIV-Tat from the cell surface. Conversely, HIV-Tat peptide abrogated AO4B08-F binding (data not shown). Confocal microscopy studies indeed suggested that AO4B08 and HIV-Tat peptide shuttle their cargoes, i.e. complexed antibodies and DNA plasmid, respectively, through the same vesicular compartments (Fig. 2C). To study the fate of HSPG during ligand internalization, cell-surface AO4B08 HS epitope abundance was determined subsequent to internalization of various HS ligands (Fig. 2D). In all cases, incubation with HS ligands resulted in a time-dependent consumption of the AO4B08 HS epitope from the cell surface; $t_{1/2}$ was between 30 and 60 min depending on the ligand (Fig. 2D). These data indicate that the same pool of cell-surface HSPG enters cells during endocytosis of AO4B08 and HIV-Tat. Importantly, induction of HSPG endocytosis by either DNA/HIV-Tat complexes or AO4B08 antibody complexes resulted in cell-surface consumption not only of the AO4B08 epitope but also of the epitope of non-internalizing RB4EA12 antibody (Fig. 2F). Furthermore, co-incubation of AO4B08 and RB4EA12 antibody complexes resulted in co-association of the two antibodies in intracellular vesicles (Fig. 2F). Together, these results suggest that the induction of endocytosis is specific for

![FIGURE 1. Epitope-specific αHS internalization.](Image 324x430)

HeLa (A) and A549 (B) cells were surface-stained at 4 °C with vsv-tagged αHS followed by mouse anti-vsv and Alexa Fluor 488-conjugated anti-mouse antibodies (gray bars). Similar experiments were performed at 37 °C to allow for antibody internalization (black bars), showing substantial internalization of clone AO4B08. Antibody complex binding and uptake were analyzed by flow cytometry as described under “Experimental Procedures.” C and D, αHS antibody uptake is through endocytosis. HeLa cells were incubated for the indicated times with AO4B08-F (red), and nuclei were counterstained with ToPro (blue) and visualized using confocal microscopy. D, HeLa cells were incubated for the indicated times with magnetic nanoparticle (100 nm) conjugated AO4B08 antibody complexes (AO4B08-M) and visualized using electron microscopy. Shown are representative pictures from at least three independent experiments. E, αHS uptake is strictly HS-dependent. Upper panels, uptake of AO4B08-F in HeLa cells following either no treatment (left panel) or pretreatment with chondroitinase ABC lyase (ABCase) or heparanase I and II lyases (HS’ase) to digest chondroitin/dermatan sulfate PG and HSPG, respectively. Lower panels, uptake of AO4B08-F in wild-type CHO-K1, pan PG-deficient pgsA-745, and HSPG-deficient pgsD-677 CHO cells as determined by flow cytometry. Filled curves, control cells incubated with mouse anti-vsv and fluorescein-labeled anti-mouse antibodies only, a.u., arbitrary units. F, AO4B08-F uptake requires 2-OS HSPG. Uptake of AO4B08-F or RB4EA12-F in wild-type (CHO-K1; light gray bars), 2-OS-deficient pgsF-17 (2-OS-def; dark gray bars), and NS-deficient pgsE-606 (NS-def; black bars) CHO cells as determined by flow cytometry. Data are presented as the mean S.D. (error bars).
the AO4B08 epitope but that the epitope of a non-internalizing antibody is co-expressed on the internalizing HSPG.

Isolation of HSPG-containing Endocytic Vesicles—To gain further insight into the nature of the internalizing HSPG, we developed a strategy for magnetic isolation of AO4B08-containing vesicles based on the ability of AO4B08 to deliver a magnetic nanoparticle-conjugated antibody (AO4B08-M; see “Experimental Procedures”) (Fig. 1D). Initial confocal microscopy studies showed that AO4B08-M and AO4B08-F were internalized to the same vesicular structures (supplemental Fig. 2A). Monitoring the fluorescence before and after magnetic isolation could thus be used to estimate the yield and purity of isolated vesicles. The abundance and intensity of fluorescent particles in the PNS of AO4B08-FIGURE 2. AO4B08 and HIV-Tat compete for HS binding, and both induce internalization of cell-surface PGs. A, cell-surface binding of Tat-Alexa Fluor 647 (2 μg/ml) in HeLa cells in the presence of increasing concentrations of AO4B08 antibody (0 – 0.2, v/v). B, uptake of Tat-Alexa Fluor 647 (2 μg/ml) over a period of 30 min in the presence of increasing concentrations of AO4B08 antibody (0 – 0.2, v/v). C, AO4B08-F and DNA/Tat complexes co-localize in endocytic vesicles. Confocal microscopy analysis of HeLa cells co-incubated for 30 min with DNA-YOYO-1/Tat complexes (10 and 40 μg/ml, respectively; green, DNA-YOYO-1) and AO4B08-F (red) is shown. D, HeLa cells were incubated with various internalizing HS ligands, i.e. AO4B08 antibody complexes (αHS), Tat peptide alone, or DNA/Tat complexes for the indicated times at 37 °C. After washing with 1 M NaCl to remove the remaining surface-bound ligands, the amount of residual cell-surface HSPG was determined by surface stain with AO4B08 antibody at 4 °C followed by flow cytometry analysis. Error bars represent S.D. F, cells were co-incubated for 30 min at 37 °C with myc-tagged AO4B08 antibody complexes (red) and vsv-tagged RB4EA12 antibody complexes (green) and visualized using confocal microscopy. Detail, magnification of the indicated area in panels to the left.

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treated cells was shown to be dependent on both temperature and time (supplemental Fig. 2B). Fluorescence microscopy (Fig. 3A) and flow cytometry (Fig. 3C) analysis of the PNS material fractionated into a magnetic and a non-magnetic fraction, demonstrating a substantial enrichment of fluorescent particles in the magnetic fraction. Electron microscopy experiments similarly showed enrichment of membrane-enclosed structures loaded with AO4B08-M in the magnetic fraction (Fig. 3B, right panel). Such structures were much less abundant in the PNS (Fig. 3B, left panel) and virtually absent in the non-magnetic fraction (data not shown). The total protein content in the magnetic fraction represented 2% of the non-magnetic fraction (Fig. 3D), further suggesting a high degree of enrichment. Negative staining transmission electron microscopy allowed improved visualization of intact, purified vesicles. Notably, after 20 min of incubation with AO4B08-M, isolated vesicles were small and unilamellar (Fig. 3E), i.e. analogous to the vesicles seen in intact cells at the same time point (Fig. 1D); a similar concordance was shown at the 1-h time point, i.e. substantially larger vesicles with internal membrane structures (cf. Fig. 1D and Fig. 3E). In summary, the magnetic purification strategy yielded a highly enriched population of intact, AO4B08-M-containing endocytic vesicles.

To determine the PG composition of isolated vesicles, cells metabolically labeled with [35S]sulfate were incubated with AO4B08-M followed by magnetic fractionation of PNS and isolation of [35S]PG. Intact [35S]PG was shown to accumulate over time in AO4B08-M-containing vesicles (Fig. 4A), and at the 1-h time point, there was an ~10-fold enrichment of [35S]PG in the magnetic fraction as compared with the starting material (PNS) and the non-magnetic fraction (Fig. 4B). High molecular weight PGs were relatively abundant in the magnetic fraction as compared with the other fractions (Fig. 4C). Similar experiments were performed with magnetic nanoparticle-conjugated antibody in the absence of AO4B08, showing insignificant nanoparticle uptake and negligible amounts of [35S]PG in the magnetic fraction, which excludes nonspecific association of [35S]PG with magnetic particles (data not shown). The vast majority of PGs from magnetic vesicles were HS-substituted, whereas as expected, PNS and non-magnetic material contained a mix of HSPG and chondroitin/dermatan sulfate PG (Fig. 5A). As shown in Fig. 5B, HSPG was enriched ~20-fold in the magnetic fraction as compared with the other fractions. These results provide biochemical evidence that intact HSPG and macromolecular cargo co-localize in endocytic vesicles.

Role of Both SDC and GPC in AO4B08-induced Endocytosis—Several HSPG core protein bands were found in AO4B08-M-containing endocytic vesicles (Fig. 5C); specifically, two bands were enriched, i.e. one band at ~60 kDa and one band at ~44 kDa. Several different HSPG core proteins were shown to be expressed in HeLa cells, at least at the mRNA level, which may not reflect actual protein expression (Fig. 5D). Core protein-specific immunoblots, however, demonstrated that GPC1 and SDC2 together with SDC1 were abundant HSPGs in HeLa cells (Fig. 5E). More importantly, the bands enriched in isolated vesicles (cf. Fig. 5C) were identified as GPC1 and SDC2, corresponding to a molecular mass of ~60 and 44 kDa, respectively. The presence of HS-substituted GPC1 as well as SDC2 in isolated vesicles was further confirmed by immunoblotting with core protein-specific antibodies (Fig. 5F). However, knock-down of either GPC1 or SDC2 did not significantly diminish AO4B08 antibody complex uptake despite efficient knock-down on both the mRNA and the protein level, suggesting a certain redundancy between the different HSPG core proteins (supplemental Fig. 3).

We next performed confocal microscopy experiments to further study vesicular co-localization of SDC and GPC with internalized AO4B08 antibody complexes. Stainings for endogenous SDC and GPC suffered from weak signals and poor specificity and were thus not further considered for co-localization experiments. Ectopically expressed SDC2-GFP and SDC3-GFP both showed strong co-association with AO4B08-F in vesicular structures (Fig. 6A). In further support of a role of both SDC and GPC, GPC3 likewise showed substantial co-localization with AO4B08-F in endocytic vesicles (Fig. 6A). Under the conditions used, overexpression of SDC or GPC had limited but differential effects on the level of AO4B08-F uptake, i.e. although GPC3 had no significant effect, AO4B08-F uptake appeared to be stimulated and inhibited, respectively, by SDC2.
and SDC3 overexpression (supplemental Fig. 4A). Together with the fact that the cell-surface abundance of the AO4B08 epitope was increased in all cases, the results imply that the HSPG core protein expression level per se does not determine the amount of AO4B08-F uptake; instead, different core proteins may have slightly different propensities for internalization (supplemental Fig. 4).

Like other GPI-anchored proteins, GPC is thought to preferentially reside in membrane raft microdomains (14), whereas transmembrane SDC is believed to be mainly non-raft-resident at steady state (30). To investigate whether GPC- and SDC-mediated endocytosis may proceed through a common vesicular compartment, AO4B08-F uptake was studied in SDC2-GFP and HA-GPC3 co-expressing cells. Antibody incubation at 4 °C was shown to co-cluster SDC2 and GPC3 at the cell surface (data not shown), and interestingly, already at 10 min of AO4B08-F uptake, triple-stained vesicles containing AO4B08-F and both SDC and GPC were clearly detectable (Fig. 6B).

**DISCUSSION**

It has long been recognized that cell-surface HSPGs are constitutively endocytosed (31, 32). However, the role of HSPG

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**FIGURE 5.** Magnetically isolated vesicles contain both SDC and GPC PGs strictly substituted with HS chains. A, HeLa cells were [35S]sulfate-labeled and incubated with AO4B08-M for 1 h as described in the legend for Fig. 4. Isolated [35S]PGs from the PNS (P) and the non-magnetic (N) and magnetic (M) fraction of PNS were either untreated (Control) or digested with heparanase I and III lyases (HS’ase) or chondroitinase ABC lyase (ABC’ase) followed by SDS-PAGE analysis. B, densitometric quantification of high molecular mass (>64 kDa) HSPGs in the respective fraction following extensive ABC lyase digestion. Data are presented as the relative amount of [35S]HSPG/µg of total protein. Mag, magnetic; Non-mag, non-magnetic. C, isolated PGs from a whole cell lysate of non-trypsinized control cells (cell lysate) or the non-magnetic and the magnetic fraction of the PNS from cells incubated with AO4B08-M as in A were digested with heparanase III and ABC lyases. The digest products were separated by SDS-PAGE, and HSPG core proteins were visualized by immunoblotting with 3G10 anti-HS antibody. Non-digested PGs (second column from the left) showed virtually no signal, confirming the specificity of the 3G10 antibody. Arrow 1, position of GPC1 (see also panel E); arrow 2, position of SDC2 (see also panel E). D, quantitative real-time PCR analysis of HSPG core protein mRNA expression in HeLa cells. Error bars represent S.D. E, immunoblotting with 3G10 anti-HS antibody and core protein-specific antibodies on purified total PG from cell extracts. F, immunoblotting with anti-GPC1 or anti-SDC2 antibodies, as indicated, of intact HSPGs purified from the magnetic fraction.
endocytosis during macromolecular delivery remains ill-defined. Here, we provide the first biochemical evidence that HSPGs are true internalizing receptors of macromolecular cargo, and for the first time, show that both SDC and GPC HSPG can mediate this transport. Furthermore, we show that αHS-induced endocytosis is epitope-specific, i.e. only one among several cell-surface-binding αHS clones was efficiently internalized. It should be emphasized that as opposed to other HS-biding ligands, e.g. growth factors and lipoproteins that may be internalized through HSPG and/or high affinity binding receptors, the strict dependence on HSPG for αHS internalization allowed us to specifically probe the HSPG endocytic pathway.

The AO4B08 antibody has shown preference for 2-OS-modified HS regions (33); accordingly, 2-OS-deficient CHO cell mutants (pgsF-17) displayed virtually no uptake of AO4B08 complexes (Fig. 1F). The structural specificity of AO4B08 uptake is quite remarkable as it has been shown that pgsF-17 mutants, despite the decrease in 2-OS, produce HS chains with normal iduronic acid content and a higher overall sulfation density as compared with wild-type CHO due to enhanced NS (27). The relative inability of the other αHS antibodies to induce HSPG endocytosis was quite intriguing. However, induction of HSPG internalization was shown to consume the HS epitope also of a non-internalizing antibody (RB4EA12) from the cell surface (Fig. 2E), and AO4B08 and RB4EA12 antibody complexes were shown to reside in common vesicular structures after co-incubation (Fig. 2F). Taken together, it is thus unlikely that the unique ability of AO4B08 to trigger macromolecular endocytosis is due to 2-OS HS epitope substitution of a specific subset of PG core proteins. Instead, the spatial distribution of AO4B08-binding HS epitopes, e.g. close to or distant from the core protein linkage region, may provide a favorable steric situation to trigger downstream HSPG clustering and membrane invagination. Notably, 2-OS deficiency in pgsF-17 cells did not affect overall turnover of HSPGs (34), thus ruling out that lack of AO4B08 uptake by this cell line is secondary to perturbed HSPG endocytosis per se.

The ability of the AO4B08 antibody to mediate magnetic nanoparticle uptake through HSPG was utilized for the isolation of intact vesicular structures. Using this approach, we show that both SDC and GPC can co-endocytose with macromolecular cargo. More importantly, our data indicate that, at an early time point (10 min) of ligand internalization, GPC and SDC can mediate this transport. Furthermore, we show that αHS-induced endocytosis is epitope-specific, i.e. only one among several cell-surface-binding αHS clones was efficiently internalized. It should be emphasized that as opposed to other HS-biding ligands, e.g. growth factors and lipoproteins that may be internalized through HSPG and/or high affinity binding receptors, the strict dependence on HSPG for αHS internalization allowed us to specifically probe the HSPG endocytic pathway.

The observation that internalized HSPG remains intact for more than 3 h in isolated vesicles (Fig. 4A) is intriguing given that GPCs are thought to be sorted for lysosomal degradation within 30 min after endocytosis and that SDCs undergo depolymerization with specific HS degradation intermediates and is degraded within 3 h (32). Our data may reflect that αHS-mediator core protein clustering directs internalized HSPGs to an alternative sorting pathway and/or that HSPG-bound antibody complexes prevent the action of endoglycosidases by steric hindrance. The latter explanation is in good agreement with the finding that iduronic acid 2-OS residues are important targets for heparanase-dependent formation of HS oligosaccharide cleavage intermediates (34). Internalized AO4B08 antibody complexes conceivably interfere with the catabolism of HS chains at several levels, and future investigations aim at a detailed structural characterization of vesicular HS.

The findings that several αHS (e.g. RB4EA12) bound to cell-surface HSPG but were not internalized and that AO4B08 was taken up by multiple cell types from various origins underline the challenge of identifying a cell- or tissue-specific HS for “targeted” drug delivery in, for example, cancer treatment. However, the term targeted could in this context have several meanings: cancer cell-specific delivery of a cytotoxic substance or non-cancer cell-specific delivery of, for example, nucleic acid-based drugs that target a pathway that is essential for cancer cell survival while dispensable for normal cells. The versatility of the HSPG pathway in αHS-mediated macromolecular transport could thus still prove important for the future development of drug delivery vehicles.

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