Heparan Sulfate Degradation Products Can Associate with Oxidized Proteins and Proteasomes*

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The S-nitrosylated proteoglycan glypican-1 recycles via endosomes where its heparan sulfate chains are degraded into anhydromannose-containing saccharides by NO-catalyzed deaminative cleavage. Because heparan sulfate chains can be associated with intracellular protein aggregates, glypican-1 autoprocessing may be involved in the clearance of misfolded recycling proteins. Here we have arrested and then reactivated NO-catalyzed cleavage in the absence or presence of proteasome inhibitors and analyzed the products present in endosomes or co-precipitated with proteasomes using metabolic radiolabeling and immunomagnet isolation as well as by confocal immunofluorescence microscopy. Upon reactivation of deaminative cleavage in T24 carcinoma cells, [35S]sulfate-labeled degradation products appeared in Rab7-positive vesicles and co-precipitated with a 20 S proteasome subunit. Simultaneous inhibition of proteasome activity resulted in a sustained accumulation of degradation products. We also demonstrated that the anhydromannose-containing heparan sulfate degradation products are detected by a hydrazide-based method that also identifies oxidized, i.e. carbonylated, proteins that are normally degraded in proteasomes. Upon inhibition of proteasome activity, pronounced colocalization between carbonyl-staining, anhydromannose-containing degradation products, and proteasomes was observed in both T24 carcinoma and N2a neuroblastoma cells. The deaminatively generated products that co-precipitated with the proteasomal subunit contained heparan sulfate but were larger than heparan sulfate oligosaccharides and resistant to both acid and alkali. However, proteolytic degradation released heparan sulfate oligosaccharides. In Niemann-Pick C-1 fibroblasts, where deaminative degradation of heparan sulfate is defective, carbonylated proteins were abundant. Moreover, when glypcan-1 expression was silenced in normal fibroblasts, the level of carbonylated proteins increased raising the possibility that deaminative heparan sulfate degradation is involved in the clearance of misfolded proteins.

Cell-associated heparan sulfate (HS)3-containing proteoglycans (PG) regulate a great diversity of biological processes involved in development, tissue repair, and tumorigenesis (1, 2). HS chains are also found both in intracellular tangles and in extracellular amyloid deposits characteristic of Alzheimer disease (3, 4). The two major cell-associated HS proteoglycan families are the syndecans and the glypicans. The syndecan core proteins are all transmembrane proteins, whereas the glypicans are attached to membrane lipids via a C-terminal glycosylphosphatidylinositol anchor (Fig. 1). The ectodomains, which carry the HS chains, vary greatly in size within the syndecan family but display considerable homology among the glypicans. The latter also share a characteristic pattern of 14 conserved Cys residues, and their HS attachment sites are concentrated to a short region near the C terminus.

Cell surface HS proteoglycans are constitutively endocytosed and degraded. Their HS chains can be cleaved into oligosaccharides by heparanase and terminally degraded by a set of exoglycosidases and sulfatases in lysosomes (5). Glypican-1 (Gpc-1), the most widely expressed member of the glypican family, is lipid raft-associated (6) and probably endocytosed via caveolae (see Fig. 1). In addition to heparanase-catalyzed degradation, Gpc-1 can undergo nitric oxide (NO)- or nitroxy (HNO)-catalyzed deaminative cleavage of its HS chains at N-unsubstituted glucosamine residues (GlCNH2) (7–9). The deaminative cleavage generates anhydromannosamine (anMan) which remains as the reducing terminal sugar of the released HS chains or oligosaccharides (10, 11). Endogenously generated anMan-positive degradation products have been detected both in cell cultures and in vivo by confocal immunofluorescence microscopy using a specific mAb (8, 9, 12, 13).

The NO/HNO required for deaminative cleavage of HS seems to be derived from preformed S-nitroso (SNO) groups in the Gpc-1 core protein. In the presence of copper ions and an NO donor, purified Gpc-1 can be S-nitrosylated in vitro (8). In vivo, copper ions can be provided by cuproproteins, such as the prion protein in fibroblasts or N2a neuroblastoma cells (14, 15), by the brain-specific glycosylphosphatidylinositol-linked splice variant of ceruloplasmin in glial cells (16), or by the Alzheimer amyloid precursor protein in neural cells (13). Accordingly,

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3 The abbreviations used are: HS, heparan sulfate; anMan, anhydromannose; anManOH, anhydromannitol; DHA, dihydroxystearic acid; DNP, 2,4-dinitrophenyl; GlCNH2, N-unsubstituted glucosamine; Gpc-1, glypican-1; Gpc-1-SNO, S-nitrosylated glypican-1; HNO, nitroxy; NPC-1, Niemann-Pick type C-1 disease; PG, proteoglycan; SNO, S-nitroso group; U18666A, 3-[2-(diethylamino)ethoxy] androst-5-en-17-one; Ub, ubiquitin; mAb, monoclonal antibody.
Gpc-1 is not S-nitrosylated in prion null fibroblasts, but S-nitrosylation is restored upon ectopic expression of the prion protein (14). No anMan-positive products are generated when prion protein lacking the copper binding domain is expressed in these cells (15). In T24 carcinoma cells, Gpc-1-SNO colocalizes with caveolin-1 (9), indicating that S-nitrosylation takes place at an early stage of recycling (see Fig. 1).

Gpc-1-SNO autodegrades its own HS chains to anMan-containing oligosaccharides when exposed to ascorbate in vitro (8). Studies on cultured cells have shown that endogenously generated anMan-positive products colocalize primarily with Rab7, a marker for late endosomes (17, 18). In Niemann-Pick C1 (NPC-1) fibroblasts, which display an endosomal transport block, staining by the anMan-specific mAb was very weak (17). However, formation of anMan-positive products that colocalized with Rab7 could be enhanced by exogenously supplied ascorbate. When N2a neuroblastoma cells were treated with 3-β[2(diethylamino)ethoxy]androst-5-en-17-one (U18666A), a compound that mimics the NPC phenotype, formation of anMan-positive products was depressed. Also in these cells, ascorbate restored formation of anMan-positive products that colocalized with Rab7. Inhibition of endosomal acidification in T24 cells, which blocks transfer from early (Rab5) to late (Rab7) endosomes, abrogated generation of anMan-positive products (18). This could also be overcome by simultaneous addition of ascorbate, which induced formation of anMan-positive products that colocalized with Rab7.

[35S]Sulfate-labeled anMan-positive products generated constitutively in T24 cells comprise free HS chains and relatively large HS oligosaccharides (18). The latter are especially prominent in Rab7-positive vesicles. Moreover, anMan-positive HS degradation products have been detected both in the cytosol (18) and in the nucleus (19), suggesting that deaminatively generated HS degradation products may exit from the endosomes (see Fig. 1).

Because HS can be associated with intracellular aggregates derived from misfolded proteins (3), we speculated that HS and its oligosaccharide degradation products might be involved in the degradation and/or clearance of misfolded recycling proteins. It has been shown that a proteasome α-subunit interacts specifically with Rab7 and thereby recruits 20 S proteasomes to multivesicular late endosomes (20). We, therefore, decided to examine if anMan-containing HS oligosaccharides can associate with misfolded proteins and proteasomes. By inhibiting and then activating deaminative cleavage in the presence of proteasome inhibitors and analyzing HS degradation products co-isolating with Rab7-positive endosomes or colocalizing with oxidized proteins or proteasomes, we have obtained results indicating that the anMan-containing HS degradation products can associate with proteins that are normally degraded in proteasomes.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—Human T24 carcinoma cells, HF-1 fibroblasts, and mouse N2a neuroblastoma cells were obtained from ATCC and human NPC-1 fibroblasts (GM03123) from the Corielle Institute. Cells were maintained in minimal essential medium supplemented with 10% fetal calf serum. Polyclonal antisera against human Gpc-1, Rab7, and Rab9 and mAbs against Rab9 and anMan-terminating HS oligosaccharides suitably tagged secondary antibodies as well as suramin, difluoromethylornithine (DFMO), U18666A, HS, N-desulfated HS, sodium l-ascorbate, enzymes, prepacked columns, and other chemicals were generated or obtained as described previously (7–9, 17, 18). A polyclonal antibody against Rab5 and a mAb against ubiquitin (Ub) were from Santa Cruz and a polyclonal antibody against the human 20 S proteasome α1 subunit was from Calbiochem. Immune-Stamp horseradish peroxidase chemiluminescent kit was purchased from Bio-Rad, and the OxyBlot protein oxidation detection kit was from Chemicon. The commercially obtained antibodies were tested by Western blotting according to the manufacturers. Proteinase K was from Sigma-Aldrich, and a proteasome inhibitor set I, including two aldehyde-terminating hydrophobic peptides, and lactacystin were obtained from Calbiochem. Polyacrylamide gels were from Invitrogen, Sweden, and protein G-Sepharose 4B was from Sigma-Aldrich. Dynabeads M-280 sheep anti-rabbit IgG was obtained from Dynal ASA, Oslo, Norway. Dehydroascorbic acid was generated by leaving a 1 M stock solution of ascorbate in contact with air for 24 h.

**Confocal Microscopy**—The various procedures including seeding of cells, fixations, the use of primary and secondary antibodies, generation of images by sequential scans, and data processing were the same as those used previously (8, 9, 17, 18). The second antibody used was either goat anti-mouse total Ig when the primary antibody was a mouse monoclonal or goat anti-rabbit IgG when the primary antibody was a rabbit polyclonal. The second antibodies were tagged with either fluorescein isothiocyanate or Texas Red and appropriately combined with secondary antibodies specific with Rab7 and thereby recruits 20 S proteasomes to multivesicular late endosomes (20). We, therefore, decided to examine if anMan-containing HS oligosaccharides can associate with misfolded proteins and proteasomes. By inhibiting and then activating deaminative cleavage in the presence of proteasome inhibitors and analyzing HS degradation products co-isolating with Rab7-positive endosomes or colocalizing with oxidized proteins or proteasomes, we have obtained results indicating that the anMan-containing HS degradation products can associate with proteins that are normally degraded in proteasomes.

**Immunoprecipitation and Separation of Radiolabeled Degradation Products**—Confluent T24 cells were preincubated with 5 mM DFMO for 24 h and then with 0.2 mM suramin and 50 μCi/ml [35S]sulfate for another 24 h as described previously (7). Cells were homogenized in phosphate-buffered saline containing 0.25 M sucrose, 0.1 M EDTA, 0.5 mM phenylmethylsulfonyl fluoride. Rab5-, Rab-7, or Rab-9 positive vesicles as well as 20 S proteasome α1 subunit-containing material were isolated from cell homogenates by using polyclonal antibodies against the various proteins as primary antibodies and Dynabeads M-280 sheep anti-rabbit IgG as magnetic secondary antibody. The magnetic particles were recovered using a magnetic particle concentrator and extensively washed with phosphate-buffered saline (at least 10 times). The radiolabeled, magnetically isolated vesicles were lysed in 0.15 M NaCl, 10 mM EDTA, 2% Triton X-100, 10 mM KH2PO4, pH 7.5, and mixed with an...
equal volume of 8 m guanidinium chloride. Lysates were then subjected to gel filtration chromatography on Superose 6 or Superdex peptide in 4 m guanidinium chloride/0.2% (v/v) Triton X-100 as described previously (7–9). 20 S proteasome immuno-isolates were directly dissolved in elution buffer and chromatographed.

For immunosialation of radiolabeled, carboxyl-containing proteins, an adaptation of the method of Hernebring et al. (21) was used. In short, confluent NPC-1 fibroblasts were incubated with 50 μCi/ml [35S]sulfate or [35S]Met/Cys for 24 h. A sucrose homogenate (1.1 ml) was treated with 150 μl of 2,4-dinitrophenyl (DNP)-hydrazine (10 x) for 15 min. After the addition of 115 μl of neutralization solution, the sample was treated with 150 μl of anti-DNP antibody (1:62) at 4 °C overnight. The immune complex was then recovered using a magnetic secondary antibody, dissolved in elution buffer, and chromatographed on Superose 6.

Co-immunoprecipitation, SDS-PAGE, and Western Blotting—Confluent T24 cells (0.5 x 10^6 cells) were preincubated with 5 mM DFMO for 24 h and then with 0.2 mM suramin and 10 μg/ml U18666A for another 24 and 16 h, respectively. Deaminative HS cleavage was reactivated by 1 mM ascorbate in the presence of proteasome inhibitors (25 μM MG-132, 25 μM proteasome inhibitor-1, and 1.5 μM lactacystin) for 4 h. In the case of N2a neuroblastoma cells, only inhibition of proteasome activity was performed.

Sucrose homogenates of the cells containing ~2 mg of protein were treated with DNP hydrazine as described above and then first treated with protein G-Sepharose 4B and then with the mAb directed against anMan-containing HS degradation products or the anti-20 S proteasome α1 subunit antibody or no antibody in the presence of added Triton X-100 (1%, v/v). Immune complexes were recovered on protein G-Sepharose 4B. The gels were washed 6 times with 0.15 m NaCl, 10 mM Tris, pH 7.4, containing 0.2% (v/v) Tween 20. Bound material was released by boiling in SDS buffer and subjected to SDS-PAGE on 4–12% gels under reducing conditions followed by transfer to blotting membrane as described (7). Membranes were blotted first with anti-DNP (1:150) then stripped in 100 mM of neutralization solution, the sample was treated with 150 μl of anti-DNP antibody (1:62) at 4 °C overnight. The immune complex was then recovered using a magnetic second-ary antibody, dissolved in elution buffer, and chromatographed on Superose 6.

**RESULTS**

Deaminatively Generated HS Degradation Products Accumulate When Proteasome Activity Is Inhibited—Previous studies have shown that treatment of T24 carcinoma cells with DFMO increases the number of GlcNH₃ residues, i.e. NO-sensitive sites, in HS of Gpc-1 (23). Hence, pretreatment with DFMO is a way to increase the yield of anMan-containing HS chains and oligosaccharides upon deaminative cleavage. Furthermore, when heparanase is inhibited by suramin, more HS is available for NO-dependent cleavage (Fig. 1).

Hence, to maximize the yield and to investigate the nature of the deaminative HS degradation products present in endosomes of T24 cells, DFMO- and suramin-treated cells were incubated with [35S]sulfate, and Rab5-, Rab7-, and Rab9-positive vesicles were immunomagnet-isolated from cell homogenates as described previously (18). The vesicles were lysed, and their contents were chromatographed on Superose 6 under dissociative conditions (4 m guanidinium chloride, 0.2% Triton X-100 at pH 5.8). As shown previously (18), a relatively homogenous pool of large [35S]sulfate-labeled oligosaccharides was present in the Rab5- and Rab7-positive vesicles but absent from the Rab9-positive vesicles. The major part of these oligosaccharides were in the Rab7-positive vesicles (Fig. 2A).

Previous studies have shown that staining for anMan-positive HS degradation products is depressed in NO-depleted T24 cells (9). This is an indirect effect caused by depletion of Gpc-1-SNO and diminished capacity for deaminative autocleavage of HS. A more direct effect is obtained with U18666A, which inhibits NO release from Gpc-1-SNO and thereby reduces formation of anMan-containing HS oligosaccharides (18). Accordingly, when DFMO- and suramin-potentreated and [35S]sulfate-labeled T24 cells were exposed to U18666A, the total yield of intracellular [35S]sulfate-labeled oligosaccharides was reduced, especially in the Rab7-positive vesicles (Fig. 2B, solid line). As shown previously (17, 18), when U18666A-treated T24 cells are subsequently exposed to ascorbate, anMan-positive staining increases. Simultaneously, a heterogeneous population of [35S]sulfate-labeled degradation products...
positive vesicles obtained from $[^{35}S]$sulfate-labeled T24 cells in the presence of proteasome inhibitors. The content of Rab7-affected deaminative HS cleavage, we reactivated this process in examine if contact between endosomes and proteasomes

C, dashed gradually declined with time (Fig. 2 black arrowheads remaining stubs (Fig. 2). The Rab9-positive endosomes transport the truncated cytosol (Fig. 2) accumulate in late endosomes, and some of them may even exit into the generating anMan-containing HS oligosaccharides. These oligosaccharides tangle (Fig. 2), probably via caveolae, is proposed to trans-

generated lipid rafts, sometimes plugged into a lipid raft (Fig. 2, black oval with two small bars). The Gpc-1 back to the Golgi, where new HS chains can be extended on the bottom left). The Rab9-positive endosomes transport the truncated Gpc-1 is depicted (Fig. 2, top left).

Deaminative cleavage is initiated by release of NO/HNO from Gpc-1-SNO in endosomes. This results in cleavage of the HS chains at the GlcNH$_3$-1-SNO can be degraded either by heparanase (an endoglucuronidase) or by an NO-catalyzed deaminative cleavage. Degradation by heparanase before deaminative cleavage will release HS oligosaccharides containing the GlcNH$_3^+$ residues (bottom right). This precludes NO-catalyzed degradation. Deaminative cleavage is initiated by release of NO/HNO from Gpc-1-SNO in endosomes. This results in cleavage of the HS chains at the GlcNH$_3^+$ residues generating anMan-containing HS oligosaccharides. These oligosaccharides accumulate in late endosomes, and some of them may even exit into the cytosol (bottom left). The Rab9-positive endosomes transport the truncated Gpc-1 back to the Golgi, where new HS chains can be extended on the remaining stubs (black arrowheads).

was recovered from the Rab7-positive vesicles (Fig. 2, B, open symbols, and C, solid line). However, the amounts obtained gradually declined with time (Fig. 2C, dashed and dotted lines).

Proteasomes can bind to Rab7-positive late endosomes, especially in the presence of proteasome inhibitors (20). To examine if contact between endosomes and proteasomes affected deaminative HS cleavage, we reactivated this process in the presence of proteasome inhibitors. The content of Rab7-positive vesicles obtained from $[^{35}S]$sulfate-labeled T24 cells treated with DFMO-, suramin-, U18666A-, and ascorbate in the presence of proteasome inhibitors was analyzed by gel chromatography on Superose 6. When proteasome activity was inhibited during a 4-h ascorbate treatment, there was a more than 10-fold increase in $[^{35}S]$sulfate-labeled products that co-precipitated with the 20 S proteasome subunit (cf. Fig. 2, E, dashed line, with F, solid line). Overall, the yield of radiolabeled products was higher in the Rab7-positive vesicles than in the 20 S proteasome subunit immuno-isolate after a 1-h ascorbate treatment but much higher in the 20 S proteasome immuno-isolate than in the Rab7 vesicles after a 4-h treatment with both ascorbate and protease inhibitors.

We then performed a wash-out chase of the radiolabeled DFMO-, suramin-, U18666A-, ascorbate-, and proteasome inhibitor-treated cells with fresh medium without ascorbate and proteasome inhibitors and analyzed radioactive products obtained from the Rab7-positive vesicles and from the 20 S proteasome subunit immuno-isolate. Surprisingly, in the Rab7-positive vesicles, the amount of $[^{35}S]$sulfate-labeled products increased markedly. Especially material eluting like HS chains increased 4–5-fold during the first hour of chase (Fig. 2D, solid and dashed lines). Also, higher molecular size material accumulated, and after 3 h of chase lower molecular size products also began to appear (Fig. 2D, dashed and dotted lines). Simultaneously, the amount of $[^{35}S]$sulfate-labeled products co-precipitating with the 20 S proteasome subunit decreased during the first hour of chase (Fig. 2F, solid and dashed lines).

In summary, the above results showed that, upon reactivation of deaminative HS cleavage in U18666A-treated T24 cells, $[^{35}S]$sulfate-labeled products appeared in Rab7-positive vesicles and co-precipitated with a 20 S proteasome subunit. Simultaneous inhibition of proteasome activity resulted in a sustained accumulation of degradation products in the Rab7-positive vesicles and even more so in the 20 S proteasome subunit immuno-isolate. Upon removal of the proteasome inhibitors, the degradation products accumulated to even greater levels in the Rab7-positive vesicles, whereas they diminished in the 20 S proteasome subunit immuno-isolate.

**Associations between Carboxyls, anMan-containing HS Oligosaccharides, and the 20 S Proteasome α1 Subunit in T24 Carcinoma Cells**—The $[^{35}S]$sulfate-labeled products present in the Rab7-positive vesicles and co-precipitating with the 20 S proteasome α1 subunit (Fig. 2) may comprise both free and protein-bound glycosaminoglycan degradation products. Deaminatively generated HS oligosaccharides contain a reducing terminal anMan residue with a reactive aldehyde, i.e. a carbonyl (Fig. 3A). Because these oligosaccharides are formed in a reductive environment (18), it cannot be excluded that the terminal anMan is reduced to anhydromannitol (anManOH), which lacks a carbonyl (Fig. 3A), although it can still be recognized by the specific mAb used in this study (12). Alternatively, the aldehyde of unreduced HS oligosaccharides reacts with amino groups in proteins forming an unstable Schiff base (Fig. 3B). By subsequent reduction or Amadori rearrangement, the linkage between oligosaccharide and protein can be stabilized (Fig. 3C).

Oxidation and carbonylation of proteins (Fig. 3) are associated with misfolding, and furthermore, oxidized protein aggregates are poor substrates for proteolysis in proteasomes (24). Hernebring et al. (21) have shown that undifferentiated embryonic stem cells contain carbonylated proteins that are elimi-
When deaminative HS degradation was reactivated by ascorbate, there was colocalization between the proteasomal marker and anMan-staining (Fig. 4, yellow arrow). The observed colocalization between anMan- and carbonyl-staining may thus partly be due to accumulation of anMan-containing HS oligosaccharides that reacted both with DNP hydrazine and with the mAb (Fig. 3A). Alternatively or in addition, there could be a colocalization between anManOH-containing HS oligosaccharides and oxidized proteins (Fig. 3A), or the colocalization could be due to formation of unstable (Fig. 3B) or stable HS-protein conjugates (Fig. 3C).

In parallel, we examined possible colocalizations between anMan reactivity and proteasomes using the polyclonal antibody to the 20 S proteasome α1 subunit (Fig. 4, D–F). When deaminative degradation was reactivated by ascorbate, there was colocalization between the proteasomal marker and anMan-staining in the same area (Fig. 4E, yellow arrow) where the carbonyl- and anMan-staining colocalized (Fig. 4B, yellow arrow). Upon simultaneous inhibition of proteasome activity, the proteasome-anMan colocalization was enhanced (Fig. 4F, yellow arrows), but some of the HS degradation products still appeared to be carbonyl-negative (Fig. 4F, green arrow). In summary, upon inhibition of proteasome activity a pronounced
colocalization between carbonyl-staining, anMan-containing HS degradation products, and proteasomes was observed, suggesting enhanced association between HS, oxidized proteins, and proteasomes.

Identification of HS Oligosaccharide-Protein Conjugates—The [35S]sulfate-labeled degradation products isolated from Rab7-positive vesicles of DFMO- and suramin-treated T24 cells comprised a relatively homogenous oligosaccharide population that was well retarded on Superose 6 (Fig. 2A). However, when deaminative cleavage was reactivated after inhibition by U18666A (Fig. 2B), most of the [35S]sulfate-labeled degradation products isolated from the Rab7-positive vesicles (Fig. 2C) as well as those co-precipitating with the 20 S proteasome α1 subunit (Fig. 2, E and F) were heterogeneous and appeared to be larger than an oligosaccharide (Fig. 2A). Because the Superose 6 chromatography was performed under dissociative conditions, the products could consist of HS oligosaccharide-protein conjugates (Fig. 3).

To isolate and analyze these radiolabeled products, we incubated T24 cells with [35S]sulfate and treated them first with DFMO to increase the number of NO-sensitive sites and with suramin and U18666A to arrest HS degradation and then with ascorbate to reactivate deaminative cleavage in the presence of proteasome inhibitors. We recovered a 20 S proteasome α1 subunit immuno-isolate, and the products associated with the proteasomes were separated on Superose 6 into two major size-pools (I and II in Fig. 5A). To test if the [35S]sulfate-labeled products in pools I and II were linked to protein, they were digested with proteinase K. After digestion, both the 35S-labeled products eluted as oligosaccharides from Superose 6 (Fig. 5, B and C) but were excluded from Superdex peptide (results not shown).

Some of the 35S-labeled products in pools I and II could be intact PG or PG with truncated side chains. If so, their glycan chains should be released by alkaline scission of the xylose-toserine bond, which connects the glycan to the protein. However, most of the products in pool I were alkali-resistant (Fig. 5D), and the same result was obtained with the 35S-labeled material from pool II (data not shown). Because aldmines (Fig. 3B) should be acid-labile, we also treated the 35S-labeled material in pools I and II with acid, but they were both resistant (data not shown).
Heparan Sulfate and Proteasomes

FIGURE 5. Characterization of [35S]sulfate-labeled products co-precipitating with the 20 S proteasome α1 subunit. T24 carcinoma cells were grown to confluence in 75-cm² dishes and radiolabeled as described under “Experimental Procedures.” The panels show gel chromatography of intact or variously degraded [35S]sulfate-labeled products present in the 20 S proteasome α1 subunit immuno-isolate obtained from DFMO-, suramin-, and U18666A-treated and [35S]sulfate-labeled T24 cells that were further treated with 1 mM ascorbate and proteasome inhibitors for 4 h. In A–D, samples were chromatographed on Superose 6, and in E–F samples were chromatographed on Superdex peptide. In A the [35S]sulfate-labeled products were separated into one high molecular size (I) and one low molecular size (II) pool. In B a portion of the [35S]-labeled products present in pool I was digested with proteinase K (I-PK) and re-chromatographed on Superose 6. In C a portion of the [35S]-labeled products present in pool II was digested with proteinase K (II-PK) and re-chromatographed on Superose 6. In D a portion of the [35S]-labeled products present in pool I was exposed to alkali (I-OH) and re-chromatographed on Superose 6. In E a portion of the [35S]-labeled products present in pool I was digested with HS lyase (I-HS’ase) and chromatographed on Superdex peptide. In F a portion of the [35S]-labeled products present in pool II was chromatographed on Superdex after deaminative degradation at pH 1.5 (II-HNO2-1.5). Cells were treated with 5 mM DFMO and 0.2 mM suramin for 24 h, with 10 μg/ml U18666A for 16 h, with 1 mM ascorbate for 4 h, and with 25 μM MG-132, 25 μM proteasome inhibitor-I, and 1.5 μM lactacystin for 4 h. Vv, void volume; Vt, total volume.

not shown), suggesting that the [35S]-labeled products were not linked to protein via aldimine bonds.

To show that the large molecular size [35S]-labeled products (pools I and II in Fig. 5A) contained HS, they were digested with HS lyase in the presence of proteasome inhibitors or treated with HNO2 at pH 1.5. HS lyase digestion of [35S]-labeled products present from pool I generated [35S]-labeled digestion products that were included on Superdex peptide (Fig. 5E), whereas undigested material eluted in the void volume (data not shown). [35S]-Labeled material in pool II, which also eluted in the void volume of Superdex peptide (data not shown), was degraded by HNO2 at pH 1.5 (Fig. 5F). Hence, both pools I and II contained HS, most likely covalently linked to protein. It cannot be excluded that the radiolabeled material also included degradation products derived from other sulfated glycosaminoglycans.

Co-immunoprecipitation of Carbonylated Proteins. 20 S Proteasomes α1 Subunit, and anMan-containing HS Oligosaccharides in T24 Carcinoma Cells—The radiolabeled HS-protein conjugates recovered from the 20 S proteasome α1 subunit immuno-isolate in the presence of proteasome inhibitors (Fig. 5A) may have been formed by a reaction between anMan-terminating HS chains or oligosaccharides and proteins, possibly damaged by oxidation. We, therefore, attempted to co-immunoprecipitate carbonylated proteins and anMan-containing HS oligosaccharides.

To capture HS-protein conjugates we pretreated T24 cells with DFMO, suramin, and U18666A. Then we activated deaminative cleavage with ascorbate in the presence of proteasome inhibitors. Cells were homogenized in sucrose-containing buffer, carbonyls were derivatized with DNP hydrazine, and products reacting either with the mAb against anMan-positive HS oligosaccharides or with the antibody against the 20 S proteasome α1 subunit in the presence of added Triton X-100 were recovered from the homogenates. These immunosulates were subjected to protein separation by SDS-PAGE, and DNP-conjugated proteins were detected by Western blotting using anti-DNP.

DNP hydrazine is expected to penetrate membranes and tag all carbonylated compounds, including oxidized proteins and anMan-containing HS oligosaccharides. In the presence of Triton X-100, DNP-tagged compounds present inside vesicles should also be accessible to immunoprecipitation.

The results showed that the same DNP-tagged proteins were present both in the proteasome and in the anMan immuno-isolates obtained from cells that were not subject to inhibition of proteasome activity (Fig. 6A). However, a 55-kDa carbonylated protein band was especially prominent in the anMan immuno-isolate. In this putative HS-protein conjugate, the carbonyl may be in the protein or in the oligosaccharide or both (see Fig. 3).

Moreover, the amount of the 55-kDa carbonylated HS-protein conjugate increased more than 2-fold, as determined by densitometry, upon reactivation of deaminative cleavage in the presence of proteasome inhibitors (Asc + Pl), whereas the other protein bands almost disappeared. After stripping and re-blotting with anti-Ub (Fig. 6B), the 55-kDa carbonyl-containing protein recovered in the proteasome immuno-isolate stained for Ub, whereas the corresponding protein recovered in the anMan immuno-isolate showed weak Ub staining.

When immunoprecipitation was performed in the absence of added Triton X-100, the same pattern of DNP-tagged proteins was obtained (result not shown). However, the 55-kDa component obtained in the anMan immuno-isolate from proteasome-
Some cells were then exposed to ascorbate in the presence of proteasome inhibitors (Asc + Pl). Cells were homogenized in sucrose-containing buffer, and carbonylated compounds were tagged with DNP hydrazine and immuno-noprecipitated with antibodies either against the 20 S proteasome α1 subunit or the anMan-containing HS oligosaccharides in the presence of Triton X-100. The immuno-isolates were subjected to SDS-PAGE (4–12% gels), and DNP- or Ub-tagged proteins, respectively, were visualized by Western blotting. Cells were treated with 5 mM DFMO and 0.2 mM suramin for 24 h, 10 μg/ml U18666A for 16 h, 1 mM ascorbate for 4 h, and 25 μg/MG-132, 25 μg proteasome inhibitor-I, and 1.5 μg lactacystin for 4 h. Membranes were first blotted with anti-DNP (A), then stripped and re-blotted with anti-Ub (B). Treatments and the migration of molecular size standards are indicated.

accumulation of carbonylated proteins in NPC-1 fibroblasts—

As shown previously (17), staining for deaminative HS degradation products by the anMan-specific mAb is greatly depressed in NPC-1 fibroblasts, suggesting that impaired transport through late endosomes also affects N-denitrosylation and subsequent deaminative HS cleavage. If carbonyl staining detects anMan-containing HS oligosaccharides, such staining should also be depressed in NPC-1 cells. On the other hand, if HS oligosaccharides are normally involved in clearance of oxidized proteins from endosomes, NPC-1 fibroblasts may accumulate carbonylated proteins.

We visualized carbonylated compounds in normal (HFL-1) and NPC-1 fibroblasts by confocal microscopy. Subconfluent HFL-1 fibroblasts, which are anMan-positive (13, 17), were also carbonyl-positive (Fig. 7A), and most of this staining colocalized with the anMan-staining both in the perinuclear area and near the cell surface (Fig. 7B, yellow/brown). There was also separate green staining suggesting that some anManOH-containing HS degradation products were formed. Treatment with U18666A to suppress deaminative cleavage did not completely abolish the carbonyl reactivity despite the disappearance of anMan-staining (result not shown). As mentioned above, the two staining procedures could have different sensitivity, but a decrease in anMan-containing oligosaccharides and a corresponding increase in oxidized proteins could also give this result. Indeed, inhibition of proteasome activity in HFL-1 fibroblasts increased carbonyl staining, some of which colocalized with the anMan-staining, whereas some of it was separate (result not shown; see silencing experiments below).

NPC-1 fibroblasts displayed intense carbonyl-staining (Fig. 7C) despite undetectable staining with the anMan-specific mAb (17). The carbonyl-staining was not visibly affected by further treatments with suramin, ascorbate, or proteasome inhibitors over a 4-h period (result not shown) despite restored formation of anMan-positive HS degradation products (17).

To identify sulfated oligosaccharides and oxidized proteins, NPC-1 fibroblasts were incubated either with [35S]sulfate or [35S]Met/Cys for 24 h. Carbonylated or proteasome-associated products were immuno-magnet-isolated by using the DNP hydrazine procedure (21) and the antibody to the 20 S proteasome α1 subunit, respectively. No [35S]sulfate-labeled oligosaccharides were recovered using the DNP hydrazine procedure (Fig. 7D, positions 40–50). However, some high molecular size material eluting in or near the void volume was obtained. This could correspond to PGs with carbonylated protein cores. In contrast, after labeling with [35S]Met/Cys, ~50-fold greater amounts of radiolabeled carbonylated products were recovered (Fig. 7E). A similar yield and Superose 6 profile were obtained when [35S]Met/Cys-labeled products were recovered using the antibody to the 20 S proteasome α1 subunit (result not shown).

effect of silencing of Gpc-1 expression on the level of carbonylated proteins in HFL-1 fibroblasts—

If the anMan-containing HS oligosaccharides that became conjugated to proteins were derived from Gpc-1, silencing of Gpc-1 expression may affect the level of carbonylated proteins. To test this, HFL-1 fibroblasts were transfected with vectors expressing either a scrambled or a Gpc-1-specific siRNA to reduce Gpc-1 expression. After 48 h, cells were homogenized in sucrose, and carbonylated compounds were tagged with DNP, recovered by immunoisolation using anti-DNP in the presence of added Triton X-100, and subjected to protein separation by SDS-PAGE. The gels were finally stained for protein (Fig. 8). A 48-h silencing of Gpc-1 expression to ~60% that of the level obtained with a scrambled siRNA resulted in an increase in carbonylated pro-
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proteins. The major protein eluting at 72 kDa increased 2-fold, as determined by densitometry (Fig. 8A).

Colocalization of anMan-Containing HS Oligosaccharides, Rab7, Rab9, 20 S Proteasome α1 Subunit, and Carbonyls in N2a Neuroblastoma Cells—Constitutive generation of anMan-positive HS degradation products in unperturbed N2a neuroblastoma cells was described in previous studies (17, 18). To investigate possible association between HS degradation products, endosomes, and proteasomes in undifferentiated N2a neuroblastoma cells, which grow in conglomerates (Fig. 9), we employed confocal immunofluorescence microscopy. When proteasomes and anMan-containing HS oligosaccharides were visualized using the polyclonal antibody to the 20 S proteasome α1 subunit, which also reacts with mouse proteasomes, and the mAb specific for anMan/anManOH-containing oligosaccharides, respectively, there was limited colocalization (Fig. 9A). However, inhibition of proteasome activity resulted in increased staining for anMan/anManOH-positive products (Fig. 9, A and B, insets) that often formed caps or clusters where they colocalized with the proteasomal marker (Fig. 9B, yellow).

Lipopolysaccharide stimulates NO formation via inducible NO synthase (25). This raises the level of protein S-nitrosylation (26), also of Gpc-1 (17, 18), which thereby increases the capacity for deaminative degradation of Gpc-1 HS. As shown above, NO release and subsequent deaminative cleavage can be induced by exogenously supplied ascorbate. However, dehydroascorbic acid (DHA) can also be used because it is taken up by many cells and converted to ascorbate in the cytosol (27). When N2a cells were exposed to both lipopolysaccharide and DHA, there was increased formation of anMan-positive products that colocalized intensely with the proteasomal marker in places where the cells made contact (Fig. 9C).

Previous studies showed that anMan-positive HS degradation products colocalize with Rab7, a late endosomal marker, in N2a cells (18). When proteasome activity was inhibited in N2a cells and staining for anMan-positive products increased (Fig. 9, D and E, insets), colocalization between Rab7 and anMan-positive products intensified, forming caps or clusters at the cell periphery (Fig. 9E). Even Rab9, a marker for endosomes transporting cargo to the Golgi, colocalized with anMan-positive products upon inhibition of proteasome activity (Fig. 9F). In the absence of proteasome inhibitors, such colocalization was limited (Fig. 9F, inset).

Treatment with DNP hydrazide, which reacts with carbonyls, yielded relatively modest staining with anti-DNP, and it colocalized almost entirely with the anMan-staining, sometimes forming clusters in the peripheral cytoplasm of N2a cells (Fig. 9, G and H, yellow and brown). This is in keeping with the presence of free unreduced HS oligosaccharides and/or stabilized HS-protein conjugates (see Fig. 3C). After treatment with proteasome inhibitors, the colocalization appeared to increase slightly (Fig. 9H). In summary, N2a cells also exhibit ascorbate- or proteasome inhibitor-induced association between anMan-containing HS degradation products, late endosomes,
and proteasomes that is detectable without pretreatments with DFMO and suramin.

**Co-immunoprecipitation of Ubiquitinated Proteins, 20 S Proteasome α1 Subunit and anMan-containing HS Oligosaccharides in N2a Neuroblastoma Cells**—Sucrose homogenates of untreated or proteasome inhibitor-treated N2a cells were exposed to DNP, and proteasome- or HS-associated proteins were isolated in the presence of added Triton X-100 as described for T24 cells and subjected to SDS-PAGE. Western blotting was performed with both anti-Ub and anti-DNP. Untreated cells yielded two major Ub-reactive proteins of ~28–30 and 60–65 kDa that co-precipitated mostly with the anti-proteasome 20 S α1 subunit but also with the mAb, detecting anMan-containing HS oligosaccharides (Fig. 10). The sharpness of the bands suggests that the proteins were not conjugated with poly-Ub.

Proteasome-inhibited cells afforded similar components, although the smaller component was not detected in the anMan immuno-isolate. However, the larger protein appeared in equal amounts in the proteasome and anMan immuno-isolates.

Stripping and re-probing with anti-DNP yielded no signal (result not shown), suggesting that there was no or little protein carbonyl formation in N2a cells. It is possible that the anMan-positive HS-protein conjugate was derived from a HS-protein conjugate stabilized by reduction (Fig. 3B). Such conjugates should be carbonyl-negative but still recognized by the anMan mAb.

**DISCUSSION**

Because HS proteoglycans are usually considered to be associated with cell surfaces and extracellular matrices, the origin of HS chains associated with intracellular, cytosolic protein aggregates (3) has been obscure. Because Gpc-1 recycles via endosomes, the HS chains could be derived from this PG. Intracellular deaminative degradation of HS in Gpc-1 appears to begin in Rab5-positive endosomes and is completed in Rab7-positive endosomes, where the bulk of the anMan/anManOH-positive HS degradation products appears (17, 18). Deaminative cleavage requires release of NO/HNO from Gpc-1-SNO, which may be S-nitrosylated during secretion at the cell surface or during endocytosis.

Traffic through the endosomal compartments is inhibited by the cationic steroid U18666A. Simultaneously, this compound suppresses generation of anMan/anManOH-containing HS degradation products (17). Upon treatment with ascorbate, anMan/anManOH-positive products reappear in Rab7-positive endosomes. The ascorbate form of vitamin C is preferentially taken up by a specific sodium-ascorbate symport, as in fibroblasts (18), whereas DHA can be taken up via the less specific hexose transporters GLUT1, -3, and -4 in other cells. Inside cells, DHA is reduced to ascorbate (27). Accordingly, exogenously supplied DHA can also activate NO-dependent degradation of HS, as in N2a cells (18).

The present results showed that when deaminative cleavage of HS is reactivated in T24 carcinoma cells, [35S]sulfate-labeled degradation products appear both in Rab7-positive endosomes and in a proteasomal subunit immuno-isolate. Sustained accumulation at these sites is obtained when reactivation of HS degradation is performed in the presence of proteasome inhibitors. Upon removal of the inhibition, there appeared to be a transfer of degradation products from proteasomes back to endosomes. To maximize the yield of anMan-containing HS degradation products, T24 cells had to be pre-exposed to both DFMO and suramin. However, in N2a cells colocalization between anMan-positive HS degradation products, Rab7-positive endosomes and proteasomes were induced when only proteasome inhibitors were added to the cell cultures.

We also demonstrate that anMan-containing HS degradation products can be detected both by a DNP hydrazide-based method (21) and by the anMan-specific mAb (12). The mAb appears to recognize anMan even when it is conjugated to DNP. Because the DNP hydrazide method also detects carbonylated proteins, colocalizations between carbonyl and anMan staining can also be due to associations between HS degradation products and oxidized, i.e. carbonylated, proteins. Indeed, when deaminative HS degradation was reactivated in T24 cells by ascorbate in the presence of proteasome inhibitors, very few free HS oligosaccharides were observed in the Rab7-positive vesicles or co-precipitating with the 20 S proteasome α1 subunit. Instead, the deaminatively generated HS degradation products were most likely covalently bound to proteins. These HS-protein conjugates were resistant to both acid and alkali. The precise nature of the covalent bonds remains to be elucidated. Both unstable and stable HS-protein conjugates may be formed (see Fig. 3).
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A carbonyl-containing, putative HS-protein conjugate of 55 kDa was recovered from T24 cells by immunoprecipitation with the mAb against anMan-containing HS oligosaccharides. The level of this component increased upon activation of deaminative cleavage in the presence of inhibitors of proteasome activity. Under these conditions the putative conjugate appeared to be present both inside as well as outside the endosomal compartment.

In N2a cells, a Ub-tagged, carbonyl-negative, 60–65-kDa protein was recovered by immunoprecipitation with the mAb against anMan-containing HS oligosaccharides, indicating that non-carbonylated proteins may also be conjugated to HS. The Rab7-proteasome–HS colocalizations were often concentrated to clusters near the cell surface, as if they were involved in exocytosis.

In NPC-1 fibroblasts traffic through the endosomal compartments is genetically defective, which results in diminished formation of anMan-containing HS degradation products (17). We show here that NPC-1 fibroblasts accumulate carbonylated proteins that co-precipitate with the proteasomal subunit. Moreover, suppression of Gpc-1 expression in normal fibroblasts increases the level of carbonylated proteins.

The present results are, thus, consistent with an involvement of Gpc-1-derived anMan-containing HS chains or oligosaccharides in the clearance of certain oxidized or non-oxidized proteins, perhaps preferentially aggregation-prone, recycling metalloproteins that are not easily degraded in lysosomes. Oxidized proteins often colocalize with proteasomes (28) in places where the anMan-containing HS degradation products also appear. The anMan-containing degradation products may form both reversible and irreversible conjugates. How HS-protein complexes are transported through the endosomal membrane to reach the proteasomes remains to be understood.

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