Basal Autophagy Is Required for the Efficient Catabolism of Sialyloligosaccharides*

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Background: The role of autophagy in glycan catabolism remains to be clarified.

Results: In Atg5−/− cells, defective in autophagosome formation, sialyloligosaccharides accumulate specifically in the cytosol.

Conclusion: Basal autophagy is essential for lysosomal catabolism of sialyloligosaccharides.

Significance: This result not only underscores the importance of autophagy in glycan catabolism but also suggests that basal autophagy is required for proper function of lysosomes.

Macronuclear autophagy is an essential, homeostatic process involving degradation of a cell's own components; it plays a role in catabolizing cellular components, such as protein or lipids, and damaged or excess organelles. Here, we show that in Atg5−/− cells, sialyloligosaccharides specifically accumulated in the cytosol. Accumulation of these glycans was observed under non-starved conditions, suggesting that non-induced, basal autophagy is essential for their catabolism. Interestingly, once accumulated in the cytosol, sialylglycans cannot be efficiently catabolized by resumption of the autophagic process, suggesting that functional autophagy is important for preventing sialyloligosaccharides from accumulating in the cytosol. Moreover, knockdown of sialin, a lysosomal transporter of sialic acids, resulted in a significant reduction of sialyloligosaccharides, implying that autophagy affects the substrate specificity of this transporter. This study thus provides a surprising link between basal autophagy and catabolism of N-linked glycans.

This novel pathway, endo-β-N-acetylglucosaminidase (8, 9) and α-mannosidase (Man2C1) (10, 11) were shown to be involved in the degradation of high mannose-type free oligosaccharides (fOSs),2 some of which are released by peptide:N-glycanase, in the cytosol of mammalian cells. On the other hand, the precise mechanism by which sialylated, complex-type glycans are catabolized in the cytosol of cells and tissues remains unclear (12, 13).

Macroautophagy (hereafter referred to as autophagy) is an intracellular bulk degradation process that delivers cytoplasmic content to lysosomes for degradation (14, 15). Proteins and other components, such as lipids or organelles, are also recycled by this pathway (16, 17). Although fOSs accumulate in the cytosol, involvement of autophagy in their degradation has not been rigorously examined.

Since the 1990s, a series of autophagy-related (ATG) genes required for the autophagic process have been identified (18, 19). Atg5 is a component of the Atg12-Atg5-Atg16L1 complex, which plays a pivotal role in the elongation and closure of the isolation membrane to form an autophagosomal (15).

In this study, we analyzed the structures and amounts of accumulated fOSs in the cytosol of mouse embryonic fibroblasts (MEFs) that lack a component required for autophagosome formation (Atg5−/−) (20). Surprisingly, we found that sialyloligosaccharides were accumulated specifically in Atg5−/− cells. Further analyses indicated that the basal autophagy process is required for keeping these sialylglycans from accumulating in the cytosol. Moreover, knockdown of sialin, a lysosomal transporter for sialic acid, in Atg5−/− cells resulted in delayed accumulation of sialyloligosaccharides in the cytosol, suggesting that the function of sialin protein is somehow compromised. Our results therefore suggest that basal autophagy is important for lysosomal function, affecting the functional properties of lysosomal proteins.

2 The abbreviations used are: fOS, free oligosaccharide; Dox, doxycycline; MEF, mouse embryonic fibroblast; PA, pyridylamino.

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**Preparation of PA-labeled Standard Glycans**—Authentic samples of PA-labeled Gn1-type (bearing only a single GlcNAc at the reducing terminus (24)) and high mannose-type glycans were prepared from various sources (25, 26). For standard Gn2-type glycans (bearing an N,N'-diacetylchitobiose structure at their reducing termini (24)), standard samples (high mannose- or complex-type glycans) were obtained from Takara Bio Inc. (Ohtsu, Japan) or Masuda Chemical Industry Co. (Takamatsu, Japan). When required, appropriate glycosidase digestion was carried out to generate new standard PA sugars. For PA-labeled, Gn1-type biantennary complex-type glycans, we first digested 25 mg of human IgG (Nihon Pharmaceutical Co., Tokyo, Japan) with 250 units of peptide:N-glycanase F (Roche Applied Science) in 1 ml of 50 mM NH₄HCO₃ at 37 °C overnight. Released glycans were collected using a Centricon™ device with an Ultracel YM-50 membrane (EMD Millipore Corp., Billerica, MA), and filtrate was desalted using a PD-10 column according to the manufacturer’s protocol. Desalted samples were evaporated to dryness. The Gn2-type biantennary complex-type glycans thus obtained were dissolved in 200 μl of distilled water, and samples were further digested with Endo F2/F3 (Sigma; 100 milliunits each) with the buffer provided at 37 °C overnight. The reaction was stopped by adding 3 volumes of ethanol, followed by centrifugation at 17,000 × g for 20 min at 4 °C. Supernatants and pellets were subjected to PA-labeling. PA-labeled Gn1-type glycans were then separated from Gn2-type glycans using an ODS column, as described previously (25). Structures of these glycans were confirmed by HPLC, MALDI-TOF MS, and glycosidase digestion analyses. When required, appropriate glycosidase digestion was carried out to generate new standard glycans.

Standard asialo-triantennary glycans (Gn1-type or Gn2-type) (i.e. Galβ1,4GlcNAcβ1,2(Galβ1,4GlcNAcβ1,4)Manα1,3(Galβ1, 4GlcNAcβ1,2Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc-PA (2/4-2 triantennary glycan; Gn2-type), Galβ1,4GlcNAcβ1,2(Galβ1, 4GlcNAcβ1,4)Manα1,3(Galβ1,4GlcNAcβ1,2Manα1,6)Manβ1, 4GlcNAc-PA 2/4-2 triantennary glycan; Gn1-type), Galβ1, 4GlcNAcβ1,2Manα1,3(Galβ1,4GlcNAcβ1,2Galβ1,4GlcNAcβ1, 6)Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc-PA (2-2/6 triantennary glycan; Gn2-type), and Galβ1,4GlcNAcβ1,2Manα1,3(Galβ1, 4GlcNAcβ1,2Galβ1,4GlcNAcβ1,6)Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc-PA (2-2/6 triantennary glycan; Gn1-type) were obtained from either bovine fetuin (2/4-2 triantennary glycans; Sigma) or human α1 acid glycoprotein (2-2/6 triantennary glycans; Sigma). Briefly, 5 mg of fetuin or α1 acid glycoprotein was first digested with *Arthrobacter ureafaciens* sialidase (10 milliunits; Roche Applied Science) in 1 ml of 40 mM sodium acetate buffer (pH 5.5) at 37 °C overnight; the desialylated protein (1 mg) was further digested with 50 units of peptide:N-glycanase F (Roche Applied Science) in 0.1 ml Tris-HCl buffer (pH 8.0) at 37 °C for overnight. After the completion of deglycosylation, the reaction was stopped by adding 3 volumes of cold ethanol followed by centrifugation (17,000 × g for 20 min at 4 °C). Supernatants thus obtained were desalted with a PD-10 column, and half of the sample was subjected to PA-labeling to obtain Gn2-type glycans. Standard samples thus obtained were purified using an amino column (25) followed by a reversed-phase column (27).
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For Gn1-type triantennary glycans, the remaining half of the free Gn2-type glycans obtained as described above was treated with 0.1 m NaOH at 50 °C for 6.5 h in order to allow conversion of Gn2-type glycans to Gn1-type (28). Samples were desalted using PD-10 and labeled with PA, and Gn1-type glycans were isolated using an ODS column (25). Our experimental conditions gave a 10–20% Gn2-to-Gn1 conversion efficiency.

HPLC Analysis—PA oligosaccharides were fractionated by various HPLC analyses. Anion exchange chromatography using a TSKgel DEAE-5PW column (7.5 φ × 75 mm; Tosoh, Tokyo, Japan) was carried out as reported previously (cf. Fig. 1A) (29). For routine quantitation of sialylated glycans, the following conditions were used (cf. Fig. 5): solvent A, 10% acetonitrile, 0.01% triethylamine; solvent B, 10% acetonitrile, 3% acetic acid, 7.4% triethylamine; flow rate, 1.0 ml/min. Elution conditions were as follows: 0–10 min, 100% A; 10–20 min, 15% solvent A, 85% solvent B. Peaks were monitored by fluorescence with λex/em = 310 nm and λem = 380 nm. The amounts of sialylated glycans were calculated based on the peak area observed from 12 to 16 min, which was subtracted from the data obtained using the sialidase-treated control. Each fraction was then quantitated using the peak area of standard PA-glucose hexamer in the PA-glucose oligomer (2 pmol/μl; Takara Bio Inc.) as a reference.

Conditions for separation of sialylated oligosaccharides on the amide-80 column were as reported previously (27). Separation and identification of PA-sugars on the amino and ODS columns were performed as described previously (25). Detailed sugar structures were determined from the elution profiles of the ODS column (expressed as glucose units), and deduced sugar structures were determined from the elution profiles of each sample. Probes for sialin, Neu2, and GAPDH used for real-time PCR were obtained from Applied Biosystems.

Western Blot Analysis—Antibodies used for Western blot analysis were as follows: mouse anti-LC3 monoclonal antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan); mouse anti-GAPDH monoclonal antibody (EMD Millipore Corp.); rabbit anti-LAMP1 antibody (Abcam, Cambridge, UK). Blotting was carried out as described previously (30) and was visualized using a LAS-3000mini system (Fujifilm Co., Tokyo, Japan).

RESULTS

Accumulation of Sialyloligosaccharides in the Cytosol of Atg5−/− MEF Cells—To examine the effect of autophagy on the catabolism of FOSs, we utilized MEF cells derived from Atg5−/−KO mice (20). It was previously reported that autophagy is unnecessary for degradation of high mannose-type FOSs, which are the oligosaccharides mainly observed in the cytosol of mammalian cells (31). This observation was consistent with our results, in which no drastic changes in the structures or amounts of these glycans was noted (Table 1). However, specific peaks for Atg5−/− cells were seen on size fractionation HPLC (Fig. 1A). We pooled the peaks and performed MS analysis and, to our surprise, saw mass numbers corresponding to sialyl-FOSs (Fig. 1B). Accumulation of sialylglycans in mammalian cell cytosol has rarely been observed (12, 13).

Our results suggest that sialyl-FOSs accumulate in Atg5−/− cells but not in wild-type MEFs. To unequivocally show that this is due to the lack of functional Atg5 protein, we utilized m5-7 cells (21), Atg5−/− isogenic cells with Atg5 gene reintroduction under a Tet-off promoter. Sialyl-FOSs, peaks of which disappeared after the sialidase treatment (third panel), were significantly reduced in Atg5-expressing m5-7 cells (Fig. 2A). In addition, when Dox was added to the m5-7 cells to impair
TABLE 1—continued
Free oligosaccharides identified in WT and Atg5−/− cells

| High mannose-type G0 glycan Abbreviation | GU | Structure | Amount (pmol/10^9 cells) | WT | Atg5−/− |
|-----------------------------------------|----|-----------|--------------------------|----|---------|
| M2B*                                   |    | Man1       | 6.74                     | 8.9| 4.7     |
| M2B                                    |    | Man1       | 6.56                     | 17 | 11      |
| M4B                                    |    | Man1       | 6.63                     | 3.7| 0.73    |
| M5A                                    |    | Man1       | 6.69                     | 2.4| 4.2     |
| M6B                                    |    | Man1       | 7.51                     | 5.0| 4.9     |
| M7A                                    |    | Man1       | 5.10                     | 1.0| 2.1     |
| M7B                                    |    | Man1       | 5.75                     | 0.66|1.0     |
| M7D                                    |    | Man1       | 6.71                     | 0.49|0.80    |
| M5A                                    |    | Man1       | 4.99                     | 1.6| 3.0     |
| M6B                                    |    | Man1       | 6.35                     | 0.82|0.77    |
| M9A                                    |    | Man1       | 5.31                     | 1.9| 2.1     |
| Total                                  | 44 |            |                          | 36 |         |

* Sialylated glycans

| SIA*                                   |    | Man1       | 4.02                     | 46 |         |
| SIB*                                   |    | Man1       | 3.22                     | 26 |         |
| SIC*                                   |    | Man1       | 3.88                     | 9.8|         |
| SIB*                                   |    | Man1       | 4.08                     | 11 |         |
| SIB                                    |    | Man1       | 6.30                     | 18 |         |
| Total                                  | 50 |            |                          |    |         |

* structures not determined.
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autophagosome formation (Fig. 2, top), time-dependent accumulation of sialyl-fOSs was also seen (Fig. 2, bottom). These results confirmed that sialyl-fOS accumulation is due to a lack of a functional Atg5 gene.

Biochemical fractionation using wild-type cells confirmed that 51% of the sialyl-fOSs were recovered in the P9 fraction (containing lysosomes; Fig. 3), whereas most of the increased sialyl-fOSs in the Atg5<sup>−/−</sup> cells were recovered from the cytosol fraction (Table 2). These results suggest that most, if not all, of the sialyl-fOSs are accumulated in the cytosol of Atg5<sup>−/−</sup> cells.

**Basal Autophagy Is Required for Efficient Catabolism of Sialyloligosaccharides**—The simplest explanation for this phenomenon (i.e. cytosolic accumulation of sialyl-fOSs under defective autophagy) is that they are specifically recruited to lysosomes via the autophagic process for degradation. To validate this hypothesis, m5-7 cells were first treated with Dox to allow accumulation of sialyl-fOSs. Autophagy was then resumed by removal of Dox. If autophagy specifically targeted sialyl-fOSs into lysosomes, one would expect them to be quickly catabolized upon resumption of autophagy. Reactivation of autophagy upon Dox removal was clearly seen, as evidenced by the occurrence of the LC3-II band (Fig. 4A). In contrast to our expectations, however, more than 70% of the sialyl-fOSs remained in the cytosol, even at 1 day after removal of Dox (Fig. 4B), whereas autophagy was reactivated within 4 h after Dox removal (21). Furthermore, it was found that reduction of sialyl-fOSs was observed for dividing cells but not for cells cultured under full confluence (i.e. non-dividing) conditions, even...
after 2 days of incubation (Fig. 4B). These results strongly indicate that clearance of sialyl-fOSs by basal autophagy, once accumulated in the cytosol, is a very slow process, and most, if not all, of the reduction is due to the dilution effect upon cell division (Fig. 4B; compare third and fourth columns). We therefore hypothesized that the autophagic process is important for proper lysosomal function (i.e. efficient lysosomal catabolism of sialylglycans) rather than the active retrieval of sialyl-fOSs from the cytosol into lysosomes.

Although we showed that Atg5/−/− cells accumulate sialyl-fOSs in an Atg5 gene-dependent manner, it is possible that this accumulation is not related to autophagy, instead representing an as yet unclarified function of Atg5 protein. To validate this possibility, we examined the MEF cells derived from Atg9a/−/−/− cells (22). Atg9a protein is involved in a distinct pathway with Atg5 and is important for the early stages of autophagosome formation (15). As shown in Fig. 5A, we also observed significant accumulation of sialyl-fOSs in Atg9a/−/−/− cells, clearly indicating that their accumulation in Atg5/−/− cells is not due to their specific function but rather is due to a general defect in the autophagy process.

Starvation-induced Autophagy Is Involved in Non-selective Catabolism of Free Oligosaccharides—Specific accumulation of sialyl-fOSs was found to occur under non-starvation conditions. To examine the effects of starvation-induced autophagy on sialyl-fOS catabolism, m5-7 cells were first cultured with Dox to shut off Atg5 expression, and autophagy was then induced by removal of Dox and concurrent incubation with starvation medium (amino acid-free DMEM) for 6 h. Sialyl-fOSs were reduced to ~60% upon starvation induction; inter-
Interestingly, cytosolic high mannose-type glycans were also reduced to a similar extent (Fig. 5B). As controls, starvation treatment alone or Dox removal in rich medium did not result in significant differences in the total amount of sialyl-fOSs and high mannose-type glycans in the cytosol of m5-7 cells. Error bars, S.D. from three independent experiments. Left, quantitation of sialyl-fOSs. Right, quantitation of high mannose-type glycans (α-mannosidase-sensitive peaks larger than that of authentic Man9GlcNAc2 glycan).

**Sialin May Be Involved in Efficient Catabolism of Sialyloligosaccharides under Normal Autophagy**—After lysosomal degradation of glycoconjugates, monomeric sialic acids have been shown to be exported out of lysosomes by a sialic acid transporter, sialin (32). This transporter is able to transport sialic acids as well as acidic sugars, such as iduronic acid or glucuronic acid (3), which suggests that substrate specificity for this transporter is rather broad. Nevertheless, sialyl-fOSs do not appear in the cytosol unless autophagy is compromised (Fig. 2A), thus suggesting that sialyl-fOSs are not substrates for this transporter under normal conditions. Because the accumulation of fOSs was found to be specific for sialylglycans, we wondered whether sialin is involved in their export from lysosomes into cytosol in Atg5−/− cells. To validate this hypothesis, sialin expression was suppressed using the shRNA technique. We successfully established two sialin knockdown cell lines with distinct shRNA constructs from m5-7 cells (73% suppression for construct 1 and 77% suppression for construct 2). We then examined the effects of sialin knockdown on sialyl-fOS accu-
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FIGURE 6. Sialin is involved in the accumulation of sialyl-fOSs in cells defective in autophagy. Top, example of anion exchange HPLC profile of the fOSs under shRNA suppression of sialin expression. Transfectants for control shRNA (top graph) and shRNA for sialin (bottom graph) were incubated in the presence of Dox to shut off Atg5 expression; after incubation for 4 days, sialyl-fOSs were quantitated. Numbers (1–4) indicate elution position of authentic mono-, di-, tri-, and tetrasialyloligosaccharides. Bottom, quantitation of cytosolic sialyl- or high mannose-type (Manx,GlcNAc-Manx,GlcNAc fractions) fOSs. Error bars, S.D. from three independent experiments. *, p < 0.05; NS, not significant versus control-transfected cells; Student’s t test.

mulation. To this end, sialin knockdown cells were tested for sialyl-fOS accumulation 4 days after the addition of Dox to shut off autophagy. Sialyl-fOS accumulation was significantly reduced for both sialin knockdown cells (Fig. 6), indicating that sialin is somehow involved in the accumulation of sialyl-fOSs in Atg5−/− cells. This also supports the notion that sialyl-fOSs originate in lysosomes.

DISCUSSION

This study clearly demonstrates a previously unknown relationship between autophagy and glycan catabolism. The effect appears to be specific to sialylglycans originating from N-glycans. Surprisingly, clearance of sialyl-fOSs upon resumption of autophagy was very inefficient, which suggests that basal autophagy is important to the proper functioning of lysosomes, thereby preventing sialyl-fOSs from accumulating in the cytosol, but the active involvement of autophagy in their catabolic pathway appears not to be significant.

There are few reports on the occurrence of sialyl-fOSs in the cytosol of mammalian cells (12, 13). Most recent studies, however, have shown that similar sialyl-fOSs are accumulated in human pancreatic (33) or prostate cancers (34) but not in normal epithelial cells, thus suggesting that these glycans can serve as useful tumor markers. The mechanisms by which these glycans are accumulated in cancer cells are not understood. It is possible that the autophagic process is involved in the accumulation of sialyl-fOSs in those cancer cells.

Our subcellular fractionation studies indicated that sialyl-fOSs accumulate in the cytosol. The cytosolic sialidase Neu2 is known to catabolize cytosolic sialyl-fOSs (13). We confirmed by quantitative PCR that Neu2 expression is, if it exists at all, very low in both WT cells and Atg5−/− MEF cells (data not shown). Neu2 enzyme is known to have very low expression in most tissues (35, 36), and it is possible that the availability of Neu2 protein may be critical for the efficient catabolism of the cytosolic sialyl-fOSs. In the absence of Neu2, however, our results also show that starvation-induced autophagy can promote catabolism of cytosolic fOSs in a structure-independent fashion, and therefore the active autophagy process can account for, at least to some extent, the catabolism of cytosolic fOSs, including sialylated ones.

Although further studies must be performed in order to unequivocally determine the source of sialyl-fOSs, our hypothesis is that they are most likely derived from lysosomes, for the following reasons: 1) in wild-type MEFs, over 50% of sialyl-fOSs are observed in the P9 fraction (containing lysosomes); 2) sialyl-fOS structures resemble those of lysosome-derived fOSs found in sialidosis patients (37, 38); and 3) down-regulation of sialin resulted in a significant reduction in the accumulation of sialyl-fOSs. Because sialin does not appear to mediate transport of
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sialyl-fOSSs under normal conditions, one could speculate that autophagy is necessary for this protein to retain strict substrate specificity; thus, only monomeric sugars can be exported out of lysosomes when autophagy is functional (Fig. 7). The detailed mechanisms, however, remain to be determined.

Irrespective of the mechanism, our data clearly indicate that autophagy is important for cells to prevent sialyl-fOSSs from accumulating in the cytosol. It is also important to note that, in our study, the autophagy process was not induced by starvation, thus suggesting that basal autophagy under non-induced conditions is important for catabolism of sialyl-fOSSs. Such basal autophagy has been described previously (39). Reportedly, defective autophagy may lead to compromised lysosomal enzyme function (40), which suggests that basal autophagy is essential for the proper function of lysosomes. Future studies will clarify how basal autophagy can contribute to the catabolism of lysosomal glycans in a structure-specific fashion.

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