Identification and Adipocyte Differentiation-dependent Expression of the Unique Disialic Acid Residue in an Adipose Tissue-specific Glycoprotein, Adipo Q*

Recently, we have shown that α2,8-linked disialic acid (diSia) residue occurs in glycoproteins more frequently than ever recognized (Sato, C., Fukuoka, H., Ohta, K., Matsuda, T., Koshino, R., Kobayashi K., Try, F. A., II, and Kitajima, K. (2000) J. Biol. Chem. 275, 15422–15431). In the course of identification of the diSia-containing glycoproteins in mammals, the 30-kDa glycoprotein was found in bovine serum. The 30-kDa glycoprotein was shown to be the bovine adipo Q, an adipocyte-specific protein, based on the partial amino acid sequences and the immuno-cross-reactivity with the recombinant mouse adipo Q. The bovine adipo Q was shown to have no N-linked but O-linked glycan(s) containing the diSia epitope, Neu5Acα2→8Neu5Acα2→3Gal. Furthermore, the diSia epitope was also found in the mouse adipo Q in serum as well as in the 3T3-L1 cells that are fully differentiated into adipocytes. Adipo Q is the diSia-containing glycoprotein and a physiological substrate of α2,8-sialyltransferase III, whose substrates have not been identified so far.

α2,8-Linked di/oligosialic acid (di/oligoSia) chains containing 2–3 Sia residues are common structural units of gangliosides and are shown to be involved in various biological functions such as cell adhesion, cell differentiation, and signal transduction (1, 2). Until recently, little attention has been paid to the existence of such short sialyl oligomers in glycoproteins, whereas the polysialic acid (polySia) chains with more than 8 Sia residues have been well studied in neural cell adhesion molecule for the regulatory functions in the cell-cell interaction during development and differentiation (3, 4) as well as in fish polysialoglycoprotein for the involvement in egg activation at fertilization (5). Recently, using the newly developed sensitive techniques (6–8), we have shown that the di/oligoSia containing up to 7 Sia residues occurs in glycoproteins more frequently than heretofore recognized and forms a new class of sialyl groups in glycoproteins (6, 8–10). This finding raised several questions as to which proteins contain the di/oligoSia groups, which enzymes are involved in the synthesis, and what is the biological significance of the modification in the glycoproteins. We have thus sought to identify the di/oligoSia-containing serum glycoproteins and the involved enzymes in the synthesis of the di/oligoSia residue of the glycoproteins. In serum glycoproteins, it has been shown that bovine fetuin and α2-macroglobulin (originally identified as a contaminant in the commercially available fetuin sample) contain the di/oligoSia structure (9).

In our further efforts to identify the di/oligoSia-containing glycoproteins in serum, in this study we clearly show that an adipose tissue-specific glycoprotein (adipo Q) contains the Neu5Acα2→8Neu5Acα2→3Gal structure in bovine and murine sera as well as in mouse 3T3-L1 cells that are differentiated into the adipocytes. Adipo Q is one of the serum glycoproteins (0.05% of total serum proteins) and is considered to play important roles in energy homeostasis (11, 12). Interestingly, adipo Q has been reported not to be a glycoprotein because of the negative sensitivity to the digestion with endo-N-acetylglucosaminidase H during the de novo synthesis of adipo Q (11). So far carbohydrate modification of adipo Q has never been described in animals. This study also indicates that the α2→8 linkage of the diSia epitope in adipo Q is synthesized by an α2,8-sialyltransferase III (ST8Sia III) (13) but not ST8Sia II (STX) (14, 15) nor ST8Sia IV (PST) (16) in the 3T3-L1-derived adipocytes. To our knowledge, this is the first identification of physiological substrate of ST8Sia III.  

EXPERIMENTAL PROCEDURES  

Materials—Clostridium perfringens sialidase, and endoproteinase Lys-C were purchased from Sigma. Arthrobacter ureafaciens sialidase was purchased from Nacalai Co. (Kyoto, Japan). Bicinchoninic acid protein assay kit was purchased from Pierce. 1,2-Diamino-3,4-methylenedioxybenzene was purchased from Dojindo (Kumamoto, Japan). Peptide-N-glycanase F was purchased from TAKARA (Kyoto Japan). DEAE-Sepharose Fast Flow (DEFF), Sephacryl S-300, Zn2+-chelating Sepharose, Sephacryl G-25, Sephadex G-50, and protein-G-Sepharose resins, enhanced chemiluminescence (ECL) reagents, and CMP-[14C]Neu5Ac (10.7 GBq/mmol) were purchased from Amersham Pharmacia Biotech. DEAE-Toyopearl 650M resins were purchased from Tosoh (Tokyo, Japan).  

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Adipo Q as a Distialic Acid-containing Glycoprotein

Japan). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). Prestained molecular marker was purchased from Bio-Rad. Peroxidase-conjugated goat anti-mouse (IgG + IgM) was purchased from American Qulex (San Clemente, CA). Peroxidase-conjugated rabbit anti-rat (IgG + IgM) was obtained from Zymed Laboratories (San Francisco, CA). Rat anti-mouse (IgG + IgM) was purchased from Cappel (West Chester, PA). Monoclonal antibody S2-566, which recognizes Neu5Ac2→8Neu5Ac→3Gal, was kindly provided by Dr. Koichi Furukawa (Nagoya University School of Medicine) and prepared as described (8). Monoclonal antibody 735, which recognizes polyNeu5Ac and OL28, which recognizes oligoNeu5Ac and polyNeu5Ac, were kindly provided by Dr. Karen Colley (University of Illinois, School of Medicine), respectively. Monoclonal antibody 1E6, which recognizes diNeu5Ac, was prepared as described previously (8). Bovine fat tissues were kindly provided by the Nagoya central wholesale market at Takahata station (Nagoya, Japan). Fetal bovine serum (FBS) was obtained from Filtron (Lot 8409, Brooklyn, Australia). Female 8-week-old animals of Balb/c mice and Wistar rats were obtained from Japan SLC Co. (Hamamatsu, Japan). Normal mouse sera were prepared from the Balb/c mice. Briefly, blood was collected and stood at 25 °C for 1 h and at 4 °C for overnight. After centrifugation at 3,000 rpm for 10 min, the supernatant was used as mouse and rat sera. Cell lysate of 3T3-L1 cells were prepared as described (8).

Characterization of Adipo Q—The adipo Q protein was prepared using affinity-based methods. In this step, the adipo Q protein was purified using established methods (8). Following the purification, the adipo Q protein was analyzed by SDS-PAGE and Western blotting. The adipo Q protein was subjected to immunoprecipitation using the anti-recombinant mouse adipo Q antibodies, as described previously (8).

Preparation of Antiserum against the Bovine 30 kDa-gp and the Recombinant Mouse Adipo Q—The bovine 30 kDa-gp (30 µg/mouse) or the recombinant mouse adipo Q (30 µg/mouse) were emulsified with Freund's complete adjuvant and injected into female Balb/c mice or male Wistar rats (female, 6 weeks old, respectively) intramuscularly. All animals were boosted twice with each protein (10 µg/mouse or 30 µg/mouse) mixed with Freund's incomplete adjuvant every 2 weeks. Blood was collected 1 week after the last boost, and the serum was prepared.

Immunoprecipitation—Mouse sera that had been pretreated with protein G-Sepharose, 3T3-L1 cell lysates, and the culture medium at day 8 were subjected to immunoprecipitation using the anti-recombinant mouse adipo Q and S2-566 antibodies that had been incubated with protein G-Sepharose as described previously (24). In the case of S2-566, protein G was pre-incubated with rat anti-mouse IgM antibodies.

Reverse Transcriptase-PCR—The following degenerate oligonucleotide primers for mouse proteins were used: STsia I (GenBank accession number X84235, nucleotides 48–1014), 5'-CAGTGGATCGAATTCT-3' and 5'-CCGCGGCGG ATGCTTC-3'; STsia II (X83562, nucleotides 46–1107), 5'-CCTCGTGGTCTCTTCGAG-3' and 5'-GCGCCTTGGAT CGTCAAGGAGGAC-3'; STsia III (X80502, nucleotides 7–1101), 5'-AGATTGCACATAGGGAGCT-3' and 5'-ATGCAGCGCTGAGCGG-3'; STsia IV (X80600, nucleotides 72–1061), 5'-AAATAGCCGAAGATGGCAGA-3' and 5'-CTCGTCGCTGAGCGG-3'; STsia V (X80914, nucleotides 358–1140), 5'-GGATGGGAAGGCTCCAGCAG-3' and 5'-CTTGCGTGGTACTAGGAGA-3'; and STsia VI (U21184, nucleotides 319–795), 5'-GGATGGAGAACG-3' and 5'-CTTGCGTGGTACTAGGAGA-3'.
Adipo Q as a Distialic Acid-containing Glycoprotein

RESULTS

Identification of the Bovine Serum 30 kDa-gp as Adipo Q—Recently, we have suggested that several glycoproteins in bovine serum contain di/oligoSia residues based on the fluorometric C7/C9 analysis, which is the highly sensitive, chemical method of detecting the α2,8-linked di/oligoSia residues (6). Among these glycoproteins, fetuin and α2-macroglobulin were first identified (9). In the course of the purification of α2-macroglobulin, it was suspected that 30 kDa-gp, which was co-purified with α2-macroglobulin on Zn2+-chelating column chromatography (9), also had the diSia residues. As shown in Fig. 1, the 30 kDa-gp was first purified from FBS. After polyethylene glycol precipitation of FBS, the precipitate was successively applied to a Zn2+-chelating column chromatography (Fig. 1a), Sephacryl S-300 gel filtration, and DEAE-Toyopearl 650M chromatography (Fig. 1b). DEAE-Toyopearl 650M chromatography resulted in two pooled fractions, A and B, as shown in Fig. 1b. The 30 kDa-gp was eluted in fraction B, whereas α2-macroglobulin was in fraction A (Fig. 1b). The 30 kDa-gp was further purified to homogeneity by subjecting the fraction B to the SDS-PAGE/electroelution (Fig. 1c). The C7/C9 analysis showed that the purified 30 kDa-gp had the diSia residues (see Table I). The yield of the 30 kDa-gp was 33 mg from 100 ml of FBS.

For identifying the 30 kDa-gp, the amino acid sequences of the N-terminal part and the Lys-C proteolytic peptide fragments were determined. The amino acid sequence from the N terminus was EDNMDPPL, and those of three internal peptide fragments that were obtained by the Lys-C digestion were ADNVNDST, GSVL, and GDQVWL. The homology search shows that exactly the same sequences as these three peptide fragments are found in the mouse adiponectin (12) and the human homologue named apm1 (26) (Fig. 2), although no identity with known proteins is found for the N-terminal sequence. It is thus suspected that the 30 kDa-gp is the bovine adiponectin Q. Since no sequence information for the bovine adiponectin Q was available, the bovine adiponectin Q was cloned by the PCR-based methods using the bovine adiponectin Q cDNA. The deduced amino acid sequence is shown in Fig. 2.

The sequence has 92 and 82% identities with the mouse and the human adiponectin Q, respectively, indicating that this cDNA clone codes for the bovine adiponectin Q. The bovine adiponectin Q consists of 240 amino acids with a secretory signal sequence at the N-terminal part (amino acids 1–17) and two potential N-glycosylation sites (Asn-45 and Asn-225). A collagenous region (amino acids 45–111) and a globular domain (amino acids 112–
been deposited in GenBank™ as accession number AF269230. The sequences of the mouse and human adiponectin with the mouse and human adiponectin (30 kDa-gp and the comparison with the mouse and human adiponectin (30 kDa-gp). The sequence of the 30 kDa-gp has been determined by the sequencing of the Lys-C proteolytic peptide fragments that are underlined. Two potential N-glycosylation sites are italicized. Amino acid sequences for the N-terminal region and the three Lys-C proteolytic peptide fragments that were determined by the sequencing are 1–17. Two potential signal peptide sequence is amino acids 18–26 in the cloned bovine adiponectin Q, indicating that the intact bovine serum adiponectin Q starts with Glu-18 (Fig. 2). This is consistent with the presumption that amino acids 1–17 are a signal peptide as in the mouse adiponectin Q (11, 12). The three peptide fragments of the 30 kDa-gp are also assignable in the bovine adiponectin Q (Fig. 2). Taken all together, it is concluded that the 30 kDa-gp is the bovine adiponectin Q. Now that the 30 kDa-gp is identified to be the bovine adiponectin Q, we call the 30 kDa-gp the bovine adiponectin Q.

**Immunoreactivity of Anti-bovine Adiponectin Q and Anti-recombinant Mouse Adiponectin Q Antibodies**—For immunochemical characterization of the bovine adiponectin Q, cross-reactivity of polyclonal antibodies raised against the bovine adiponectin Q (30 kDa-gp) and the recombinant mouse adiponectin Q was tested. Three antisera raised against the bovine 30 kDa-gp in three mice designated MA1, MA2, and MA3 were tested for the reactivity with the recombinant mouse adiponectin Q, a 51-kDa glutathione S-transferase fusion protein of mouse adiponectin Q expressed in *Escherichia coli*. MA1 and MA3 were reactive with the recombinant mouse adiponectin Q, whereas MA2 was not. Fig. 3a shows the results for MA3. This cross-reactivity with the recombinant mouse adiponectin Q (Fig. 3a, lane 2) is consistent with the conclusion that the bovine serum 30 kDa-gp is the bovine adiponectin Q. Furthermore, two antisera against the recombinant mouse adiponectin Q, designated RA1 and RA2, were generated. RA2 had the cross-reactivity with the bovine adiponectin Q (Fig. 3b, lane 1), whereas RA1 did not (data not shown). The cross-reactivity of RA2 shows that the bovine adiponectin Q shares the common epitopes with the mouse adiponectin Q, which is also consistent with the above conclusion.

The bovine adiponectin Q was eluted at the pass-through fraction on Sephacryl S-300 chromatography (data not shown), indicating that the bovine adiponectin Q is larger than 150 kDa and is present as homooligomers of the 30-kDa subunit, as were in the cases with other animal adiponectin Q (11, 27). The bovine adiponectin Q gave a 30-kDa band exclusively and a minor 20-kDa band on SDS-PAGE under the reducing conditions, whereas it gave a prominent 60-kDa band and minor 90- and 120-kDa bands under the nonreducing conditions (Fig. 3a, lane 3), as was reported for the mouse adiponectin Q (11).

**The Bovine Serum Adiponectin Q Contains the diSia Structure**—To examine if modification of adiponectin Q with the diSia epitope is confined to the bovine adiponectin Q, we also characterized the mouse serum adiponectin Q. The mouse adiponectin Q was immunoprecipitated from mouse sera using the anti-recombinant mouse adiponectin Q antibody RA1 and analyzed by Western blotting using S2-566. As shown in Fig. 5a, mouse adiponectin Q was precipitated from mouse sera (upper panel), and the immunoprecipitated bovine serum adiponectin Q as a Disialic Acid-containing Glycoprotein.

![Fig. 3](http://www.jbc.org/) Immuno blotting of the bovine 30 kDa-gp and the recombinant mouse adiponectin Q antibodies. a, the mouse anti-bovine 30 kDa-gp antiserum MA3, which recognizes both bovine and mouse adiponectin Q (1:1000 dilution). b, the rat anti-recombinant mouse adiponectin Q, RA2, which recognizes bovine and mouse adiponectin Q (1:1000 dilution). 0.2 µg of each of the bovine 30 kDa-gp antiserum MA3 or MA2 (Fig. 3a, lane 1) and under nonreducing conditions (lane 1) and under nonreducing conditions (lane 3) and 0.12 µg of the mouse recombinant adiponectin Q (51 kDa) (lane 2) were electrophoresed and immunostained as described under “Experimental Procedures.” The band of 60 kDa is a dimer of the 30 KDa-gp (a, lane 1).
Adipo Q immunopurified from sera and the 3T3-L1 cells. Anti-mouse adipo Q (RA1) and S2-566 after it was treated with sialidase from mouse 3T3-L1 cells, although more components were detected for the differentiated cells than the undifferentiated cells (Fig. 6b, IE6). S2-566 also visualized several components for the differentiated 3T3-L1 cells (Fig. 6b, S2-566). The intensity and number of the S2-566-reactive bands increased at the 20–45-kDa region after differentiation, consistent with the increase in the CP value in the same region (Fig. 6b, diSia). No component was detected by anti-polySia antibody (735) or anti-oligoSia antibody (OL28) (Fig. 6b, polySia and oligoSia), suggesting that the oligo/polySia chain with more than four Sia residues is not present in glycoproteins of 3T3-L1 cells, as estimated by the immunospecificity of these antibodies. All these results indicate that 3T3-L1 cells have the ability to synthesize the diSia residues in various glycoproteins of the cells, including adipo Q (Fig. 5b), and the amounts of diSia epitope were higher in the fully adipocyte-differentiated cells than in the undifferentiated cells.

Expression of α2,8-Sialyltransferases in 3T3-L1 Cells during Differentiation—To gain an insight into the biosynthesis of the diSia structure of adipo Q in 3T3-L1 cells, the expression of mRNA for the known α2,8-sialyltransferases, including ST8Sia I and V, which only utilize glycolipids as sub-

**Fig. 4. Immunostaining of the bovine adipo Q (30 kDa-gp) with monoclonal antibody S2-566.** The bovine adipo Q (1 µg per lane) was subjected to SDS-PAGE under reducing conditions and blotting to the PVDF membrane. The membrane was treated (+) or untreated (−) with sialidases (0.5 unit/ml) (a) and peptide:N-glycosidase F (PNGase F) (50 milliunits/ml) (b) at 37 °C for 20 h before immunostaining with S2-566 (0.51 µg/ml) as described under “Experimental Procedures.”

**Fig. 5. Immunodetection of the diSia epitope in the mouse adipo Q as a Disialic Acid-containing Glycoprotein in 3T3-L1 Cells before and after Differentiation—3T3-L1 cells were harvested, homogenized, and subjected to SDS-PAGE followed by blotting on the PVDF membrane. The membrane was cut into 11 equal pieces according to the descending molecular mass region and analyzed for diSia by the fluorometric C9/C7 method. The results of the fluorometric C9/C7 analysis are shown as molar proportions of internal to total Sia residues (C9/C7) and as apparent <DP> values in Fig. 6a. After differentiation, the C9/C7 value increased especially at 20–45 and >120 kDa after differentiation. This suggests that the relative amount of diSia-to-monomialyl structure increases in glycoproteins after differentiation. The results of Western blotting are shown in Fig. 6b. The anti-diSia antibody (1E6) visualized several glycoproteins of both differentiated and undifferentiated 3T3-L1 cells, although more components were detected for the differentiated cells than the undifferentiated cells (Fig. 6b, IE6). S2-566 also visualized several components for the differentiated 3T3-L1 cells (Fig. 6b, S2-566). The intensity and number of the S2-566-reactive bands increased at the 20–45-kDa region after differentiation, consistent with the increase in the CP value in the same region (Fig. 6b, diSia). No component was detected by anti-polySia antibody (735) or anti-oligo + polySia antibody (OL28) (Fig. 6b, polySia and oligoSia), suggesting that the oligo/polySia chain with more than four Sia residues is not present in glycoproteins of 3T3-L1 cells, as estimated by the immunospecificity of these antibodies. All these results indicate that 3T3-L1 cells have the ability to synthesize the diSia residues in various glycoproteins of the cells, including adipo Q (Fig. 5b), and the amounts of diSia epitope were higher in the fully adipocyte-differentiated cells than in the undifferentiated cells.

**Expression of α2,8-Sialyltransferases in 3T3-L1 Cells during Differentiation—To gain an insight into the biosynthesis of the diSia structure of adipo Q in 3T3-L1 cells, the expression of mRNA for the known α2,8-sialyltransferases, including ST8Sia I and V, which only utilize glycolipids as sub-

**TABLE I**

| Internal Sia | Terminal Sia |
|--------------|-------------|
| mol          | mol         |
| 0.62         | 0.78        |

**Expression of 2,8-Sialyltransferases in 3T3-L1 Cells during Differentiation—To gain an insight into the biosynthesis of the diSia structure of adipo Q in 3T3-L1 cells, the expression of mRNA for the known 2,8-sialyltransferases, including ST8Sia I and V, which only utilize glycolipids as sub-

**Adipo Q Secreted from Adipocyte-differentiated 3T3-L1 Cells Is Modified by the diSia Structure—**It has been shown that the mouse adipo Q is biosynthesized in and secreted from mouse 3T3-L1 cells on the treatment with differentiation-inducing reagents (12). The 3T3-L1 cells were harvested at 0, 2, and 8 days after the differentiation induction, and thecell lysates and the culture supernatant and the cell lysates were subjected to immunoprecipitation by the anti-recombinant mouse adipo Q antibody RA1 or anti-diSia antibody S2-566. These precipitates were analyzed by SDS-PAGE/Western blotting using RA1 (Fig. 5b). As shown in Fig. 5b (upper panel), adipo Q was clearly detected in the immunoprecipitate by RA1 from the cell lysates and the culture supernatant at day 8 of differentiation, whereas faint stains and no stains of adipo Q were observed at days 2 and 0, respectively. Since RA1 does not recognize the bovine adipo Q, the stained bands are not derived from the bovine adipo Q that might be contaminated from the culture medium containing FBS. These results are consistent with the results of the expression of adipo Q mRNA (see Fig. 7b). Immunoprecipitated adipo Q both from the cell lysates and the culture medium was immunostained with S2-566 (Fig. 5b, lower panel). Eight days after differentiation, the cell lysates were subjected to immunoprecipitation by S2-566 followed by Western blot analysis using RA1, and the S2-566 was shown to immunoprecipitate the 30 kDa-gp that was recognized by RA1 (data not shown). It is thus suggested the mouse adipo Q synthesized in and secreted from adipocyte-differentiated 3T3-L1 cells contains the diSia epitope.

**Chemical and Immunochemical Detection of diSia-containing Glycoproteins in 3T3-L1 Cells before and after Differentiation—**3T3-L1 cells were harvested, homogenized, and subjected to SDS-PAGE followed by blotting on the PVDF membrane. The membrane was cut into 11 equal pieces according to the descending molecular mass region and analyzed for diSia by the fluorometric C9/C7 method. The results of the fluorometric C9/C7 analysis are shown as molar proportions of internal to total Sia residues (C9/C7) and as apparent <DP> values in Fig. 6a. After differentiation, the C9/C7 value increased especially at 20–45 and >120 kDa after differentiation. This suggests that the relative amount of diSia-to-monomialyl structure increases in glycoproteins after differentiation. The results of Western blotting are shown in Fig. 6b. The anti-diSia antibody (1E6) visualized several glycoproteins of both differentiated and undifferentiated 3T3-L1 cells, although more components were detected for the differentiated cells than the undifferentiated cells (Fig. 6b, IE6). S2-566 also visualized several components for the differentiated 3T3-L1 cells (Fig. 6b, S2-566). The intensity and number of the S2-566-reactive bands increased at the 20–45-kDa region after differentiation, consistent with the increase in the C9/C7 value in the same region (Fig. 6b, diSia). No component was detected by anti-polySia antibody (735) or anti-oligo + polySia antibody (OL28) (Fig. 6b, polySia and oligoSia), suggesting that the oligo/polySia chain with more than four Sia residues is not present in glycoproteins of 3T3-L1 cells, as estimated by the immunospecificity of these antibodies. All these results indicate that 3T3-L1 cells have the ability to synthesize the diSia residues in various glycoproteins of the cells, including adipo Q (Fig. 5b), and the amounts of diSia epitope were higher in the fully adipocyte-differentiated cells than in the undifferentiated cells.**
strates, ST8Sia II (STX) and ST8Sia IV (PST), which utilize glycoproteins, and ST8Sia III, which utilizes both glycoproteins and glycolipids, was examined by the reverse transcriptase-PCR method. Only the ST8Sia III mRNA was detected at any stages of differentiation (Fig. 7a, days 0, 2, and 8), whereas four other mRNAs were not detected even after 40 cycles of amplifications. As controls, we used mouse adult brain, where the low expression of the ST8Sia II and IV mRNAs and the high expression of ST8Sia I, III, and V mRNAs are reported (28), and these results were reproduced as shown in Fig. 7a. Semi-quantitative reverse transcriptase-PCR analysis suggested that expression of the adipo Q mRNA was prominent at day 8 (Fig. 7b), as reported previously (11, 12). This expression profile of the adipo Q mRNA is coincident with protein expression of adipo Q as shown in Fig. 5b. On the other hand, the expression level of ST8Sia III mRNA of days 2 and 8 was 1.3 times higher than that of day 0 (Fig. 7b and c). These results suggest that ST8Sia III, but not other known sialyltransferases, is involved in the synthesis of the diSia epitope of the mouse adipo Q.

**DISCUSSION**

Recently, it has been demonstrated that di/oligoSia-containing glycoproteins occur in nature more frequently than ever recognized, as analytical methods for detecting the di/oligoSia structures have been improved (8). In this study, we identified adipo Q from bovine and mouse sera as a new member of the diSia-containing glycoproteins using the chemical and immunochemical methods. Bovine adipo Q has been cloned and found to consist of 240 amino acids with a secretory signal sequence (amino acid 1–17) followed by collagenous (amino acids 45–111) and globular domains (amino acids 112–240) like the mouse (12) and human adipo Q (26).
The bovine adipo Q gives a band of 30 kDa on SDS-PAGE under the reducing conditions, whereas it gives bands at 60, 90, and 120 kDa under the nonreducing conditions (Fig. 3a, lane 3), and it is eluted at >150 kDa on Sephacryl S-300 chromatography (data not shown). These results suggest that the bovine adipo Q forms oligomers of the 30-kDa subunit. All of these features for the bovine adipo Q are common with those of the mouse adipo Q (11, 12, 27). It has been believed that the mouse adipo Q is not a glycoprotein, because endo-N-acetylglucosaminidase H treatment has no effect on the molecular mass of the molecule at any stages of the biosynthesis (11). For the bovine adipo Q, it is suggested that the N-linked glycan chain does not exist. Of two potential N-glycosylation sites (Asn-45 and Asn-225), at least Asn-255 is not glycosylated because Asn was detected by the amino acid sequence analysis (Fig. 2). Furthermore, the adipo Q was not susceptible to peptide:N-glycanase F treatment (Fig. 4b). However, the fluorometric C7/C9 analysis and Western blot analysis using monoclonal antibody S2-566 indicate that the bovine and mouse serum adipo Q contain the diSia structure (Figs. 4 and 5a). We have also shown that the bovine adipo Q contains carbohydrates (Man, Glc, GlcNAc, Gal, and Sia) by the gas-liquid chromatography and fluorometric HPLC analysis. It is thus concluded that adipo Q has the diSia epitope most possibly on its O-linked glycan(s). Adipo Q is secreted by 3T3-L1 cells that are differentiated into adipocytes under insulin control (12). Here we show that the mouse adipo Q secreted from the adipocyte-differentiated 3T3-L1 cells also has the diSia epitope (Fig. 5b). The presence of the diSia epitope in adipo Q not only from serum but also from the adipocyte-differentiated 3T3-L1 cells suggests the ubiquitous modification of adipo Q with the diSia epitope.

Adipo Q is a serum protein (0.05% of total serum protein) secreted from adipose tissue (11, 12). The mouse adipo Q is also designated as ACRP30 (adipocytel complement-related protein 30), and its human homologue is designated as apm-1 (adiponectin) (26) or GBP28 (27). Adipo Q is considered to play an important role in energy homeostasis because the adipo Q mRNA decreases in adipose tissue in both ob/ob mice and obese humans (12). The three-dimensional structure of its C-terminal globular domain has close structural homology to tumor necrosis factor-α (29), which is also secreted by adipocytes and implicated in insulin resistance and energy control (30). Recently, it has been shown that human adiponectin appears to dysregulate the growth of myelomonocytic progenitors and the function of macrophages (31) and accumulation of macrophages when endothelial barrier is injured (32). Now that adipo Q is shown to have the diSia epitope on the molecule, it is conceivable that this glycoconjugate may be functionally involved in these physiological phenomena. Elucidation of biological functions of the diSia epitope of adipo Q is under way in our laboratory. It should be noted that a leptin-binding protein, designated OB-BP2, has the ability to bind the α2,8- and α2,3-Sia residues of glycoproteins and glycolipids (33). OB-BP2 is structurally classified as a member of siglec (sialic acid binding immunoglobulin-like lectin) and is named siglec-5 (34). OB-BP2 has separate binding sites for the Sia residues and leptin on the molecule. Considering that the expression of adipo Q is related with obesity (11, 12) like leptin, it would be interesting to see if the diSia-containing adipo Q binds leptin through OB-BP2 to regulate the leptin-mediated energy homeostasis.

We also show that several glycoproteins other than adipo Q also have the diSia epitope in adipocyte-undifferentiated and differentiated mouse 3T3-L1 cells by the fluorometric C7/C9 analysis and the immunoreactivity with the anti-diSia antibodies (Fig. 6, a and b). It appears that the diSia-containing glycoproteins increase after differentiation (Fig. 6b). These results suggest that 3T3-L1 cells are useful for biosynthetic study of the diSia epitope of these glycoproteins. So far, no enzyme involved in the synthesis of diSia epitope in glycoproteins has ever been identified. The ST8Sia III is a candidate enzyme for diisialylation because the enzyme has been shown to catalyze the synthesis of the diSia epitope in both glycoproteins and glycolipids in vitro (13), although the physiological substrates of this enzyme have remained unidentified. To identify the enzyme responsible for the synthesis of the diSia epitope, we sought it in 3T3-L1 cells that not only secrete the diSia-containing adipo Q but also express several diSia-containing glycoproteins (Fig. 6). Interestingly, of known five α2,8-sialyltransferases, only ST8Sia III was detected in the 3T3-L1 cells. This strongly suggests that ST8Sia III is involved in the synthesis of the diSia epitope on various glycoproteins including adipo Q in 3T3-L1 cells. The semi-quantitative analysis of mRNAs for ST8Sia III and adipo Q in 3T3-L1 cells shows that ST8Sia III is expressed constitutively, although its expression is 1.3-fold higher after differentiation. On the other hand, adipo Q is expressed only after differentiation. Therefore, the synthesis of the diSia epitope on adipo Q in 3T3-L1 cells appears to be regulated by the expression level of ST8Sia III. We also identified mRNA for ST8Sia III in mouse fat tissues. Furthermore, it is shown that adipo Q purified from bovine serum is sialylated by the mouse recombinant ST8Sia III. All these results indicate that adipo Q is a physiological substrate of ST8Sia III. To our knowledge, this is the first example of physiological substrates for ST8Sia III.

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