Intracellular Distribution of Lysosomal Sialidase Is Controlled by the Internalization Signal in Its Cytoplasmic Tail*

Received for publication, May 18, 2001, and in revised form, September 19, 2001
Published, JBC Papers in Press, September 24, 2001, DOI 10.1074/jbc.M104547200

Kiven E. Lukong‡‡, Volkam Seyranterepe§§, Karine Landry‡, Stéphanie Trudeau‡, Ali Ahmad‡, William A. Gaht‖, Stéphane Lefrancois‖, Carlos R. Morales‖, and Alexey V. Pshezhetsky‡***

From the ‡Hôpital Sainte-Justine and Département de Pédiatrie, Université de Montréal, Montréal, Québec H3T 1C5, Canada, the †Department of Anatomy and Cell Biology, McGill University, Montréal, Québec H3A 2B2, Canada, and the ¶Heritable Disorders Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Sialidase (neuraminidase), encoded by the neu-1 gene in the major histocompatibility complex locus catalyzes the intralysosomal degradation of sialylated glycoconjugates. Inherited deficiency of sialidase results in sialidosis or galactosialidosis, both severe metabolic disorders associated with lysosomal storage of oligosaccharides and glycopeptides. Sialidase also plays an important role in cellular signaling and is specifically required for the production of cytokine interleukin-4 by activated T lymphocytes. In these cells, neu-1-encoded sialidase activity is increased on the cell surface, suggesting that a specific mechanism regulates sorting of this enzyme to the plasma membrane. We investigated that mechanism by first showing that sialidase contains the internalization signal found in lysosomal membrane proteins targeted to endosomes via clathrin-coated pits. The signal consists of a C-terminal tetrapeptide 412YGTL415, with Tyr412 and Leu415 essential for endocytosis of the enzyme. We further demonstrated that redistribution of sialidase from lysosomes to the cell surface of activated lymphocytes is accompanied by increased reactivity of the enzyme with anti-phosphotyrosine antibodies. We speculate that phosphorylation of Tyr412 results in inhibition of sialidase internalization in activated lymphocytes.

Lyosomal sialidase (neuraminidase), encoded by the neu-1 gene in the major histocompatibility complex locus catalyzes the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins, and glycolipids. In the lysosome, sialidase is associated with lysosomal carboxypeptidase A (also cathepsin A or protective protein), β-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase in a multienzyme complex (1–3). Dissociation of the complex in vitro results in complete inactivation of sialidase, although activity can be restored after the reconstitution of the complex (2). These results suggest that association with the complex is required for sialidase to adopt its catalytically active conformation, but direct structural data are not available to support this mechanism. Inherited mutations in cathepsin A result in disruption of the complex and cause galactosialidosis, an autosomal recessive disease characterized by combined deficiency of sialidase, β-galactosidase, and cathepsin A (4). Another autosomal recessive disease, sialidosis, is caused by mutations directly affecting the lysosomal sialidase gene (5).

Various data obtained during the purification of sialidase from different tissues have demonstrated the existence of two pools of lysosomal sialidase, soluble and membrane-associated. Both forms appear to be encoded by a single gene, since they are absent from cultured cells of sialidosis patients (6, 7). Using immunoelectron microscopy, we have demonstrated the presence of sialidase on lysosomal membranes and within the lumen of lysosomes, as well as on plasma membranes of transfected cells (8). In addition, activated T lymphocytes exhibit severalfold increase of sialidase activity on their cell surfaces (9, 10). Lyosomal sialidase and the sialidase expressed on the cell surface are products of the same gene, since cell surface expression does not occur in T-cells obtained from SMJ or SM/B10 mice that have mutation in the neu-1-encoded sialidase (11). These data suggest that a specific mechanism exists for sorting newly synthesized sialidase to the plasma membrane and the lysosome, as well as for retention of sialidase on the plasma membrane.

Previous studies have described several pathways for sorting lysosomal membrane proteins and endocytosed receptors to the trans-Golgi network (TGN),1 plasma membrane, and endosome. All these cargo proteins contain amino acid motifs with conserved tyrosine or dileucine residues recognized by adaptor protein (AP) complexes. These coat proteins form vesicles destined for the lysosome (12–14). Two adaptor complexes, AP1 and AP2, are associated with clathrin-coated vesicles derived from the Golgi and the plasma membrane, respectively. Both AP1 and AP2 contain μ-subunits (μ1 and μ2, respectively) that recognize and bind to the internalization signals of their cargo proteins (15). The AP3 adaptor complex is required for the formation of lysosomal/endosomal vesicles from the TGN. Proteins targeted to these vesicles contain tyrosine-based internalization signals, and genetic deficiency of AP3 in the cells of patients suffering from Hermansky-Pudlak syndrome (HPS) results in increased surface expression of such proteins (16).

In this study we obtained direct evidence that sialidase is synthesized and transported to the lysosomes as a membrane-bound protein. We show that the C terminus of sialidase contains a tyrosine-based internalization signal represented by

---

* This work was supported by Canadian Institutes of Health Research Operating Grant MT-15079, Vaincre les Maladies Lysosomales Foundation, as well as an equipment grant from Canadian Foundation for Innovation (to A. V. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to the results of this work.

‡ To whom correspondence should be addressed: Service de génétique médicale, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine, Montréal (Qc) H3T 1C5, Canada. Tel.: 514-345-4931, ext. 2736; Fax: 514-345-4801; E-mail: alex@justine.umontreal.ca.

§ Both authors contributed equally to the results of this work.

¶ The abbreviations used are: TGN, trans-Golgi network; AP, adaptor protein; HPS, Hermansky-Pudlak syndrome; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; CTLA-4, cytotoxic T lymphocyte-associated antigen-4.
tating the essential Tyr 412 and Leu 415 residues.

In particular, TAT codon of Tyr 412 was changed to GCT, GGG codon of primers A and D to obtain the product containing the desired mutation. Mutagenic sense primer B and antisense primer D (Table I). Fragments with sense primer A and mutagenic antisense primer C and BD with were generated by site-directed mutagenesis using a overlap-extension

Vectors using LipofectAMINE Plus reagent (Life Technologies, Gaithersburg, MD) as described (20). Sialidase, N-acyethyl-β-hexosaminidase, β-galactosidase, and β-glucosidase activities were assayed in cellular homogenates using the corresponding 4-methylumbelliferyl (muf)-glycoside substrates as described (21–24). Cathepsin A activity was deter-

mined with CBZ-Phe-Leu (28). Cathepsin B activity was assayed with Z-Arg-Arg-pNA (25). Alkaline phosphatase activity was measured with the assay kit (Sigma) using the manufacturer’s protocol.

Solubilization of Sialidase Expressed in COS-7 Cells—COS-7 cells were co-transfected with the wild-type human sialidase and cathepsin A (15). After 48 h, adherent cells were harvested, washed with PBS, resuspended in 1 ml of ice-cold 50 mM sodium acetate buffer (pH 5.2) and sonicated by 3 pulses, 5 s each at 50 W. After centrifugation for 1 h at 100,000 × g, the supernatant (S1) was collected and the pellet was resuspended in 1 ml of 50 mM sodium acetate buffer (pH 5.2), containing 1% (v/v) of 200-fold compared with the homogenization of the cells and the presence of primary anti-sialidase antibodies diluted in Dulbecco’s modified Eagle’s medium, containing 10% (v/v) of fetal bovine serum and 25 mM HEPES (pH 7.4). Subsequently the cells were washed 3 times 5 min each with ice-cold PBS, fixed in 3% paraformaldehyde, blocked, permeabilized, and stained with Oregon Green 488-conjugated anti-rabbit IgG and Oregon Green 488-conjugated anti-mouse IgG antibodies (Molecular Probes, Eugene, OR). Slides were studied on a Zeiss LSM410 inverted confocal microscope (Carl Zeiss Inc., Thornwood).

Antibody Internalization Assay—Antibody internalization assay was performed as described (16). Normal and HPS 2 fibroblasts grown on glass coverslips were incubated for 15 min at 37 °C in the presence of a protease inhibitor mixture (Roche Molecular Biochemicals). The same procedure was used for the purification of lysosomal membranes from COS-7 cells, but the glass and glass Potter-Elvehjem homogenizer was used for the homogenization of the cells and during the first centrifugation step the speed was reduced to 1000 × g. The lysosomal membranes were purified 150–200-fold compared with the homogenate as determined by the increase of specific activity of the marker enzyme, β-glucosidase, and were not cross-contaminated by microsomal (marker enzyme, UDP-galactosyl transferase) or plasma (marker enzyme, alkaline phosphatase) membranes. The average yield (measured as the total activity of β-glucosidase recovered in the lysosomal fraction) was about 20–30% as compared with the homogenate.

Sialidase Activity Based Internalization Assay—For the purification of lysosomal membranes, 48 h after transfection, COS-7 cells from one T-25 flask were washed with Hank’s balanced salt solution and then scraped in the presence of ice-cold homogenization buffer. Cells were homogenized by 20 strokes in glass and glass Potter-Elvehjem homogenizer and the procedure for purification of lysosomal membranes followed as described above. The homogenization was performed at reduced speed (1000 g versus 3000 g) during the first centrifugation step.

Plasma membranes were purified as described (26) by binding COS-7 cells to Cytodex 1 beads (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada). The preparation was enriched ~80-fold by following the specific activity of alkaline phosphatase and did not contain lysosomal marker enzymes (β-hexosaminidase, β-galactosidase, or β-glucosidase). To measure the sialidase activity on the outer cell surface, the cells seeded in 6-well culture dishes were washed several times with Hank’s balanced salt solution and overlayered with 500 μl of 20 mM acetate buffer (pH 5.2), 0.25 M sucrose, and 0.4 mM sialidase substrate, MuNANA. After 30 min of incubation, 200-μl aliquots of medium were added to 1.8 ml of 0.4 M glycine buffer (pH 10.4), and the concentration of the product was measured as described (21). To measure the total amount of enzyme, the assay was performed in the presence of 0.3% Triton X-100.

Western Blotting of COS-7 Cells—Proteins were resolved by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27) and electropheretor to polyvinylidifluoride membrane. The sialidase and cathepsin A detection was performed with rabbit antibodies as described (28) and LAMP-2 detection with monoclonal antibodies against LAMP-2 antibodies (Worthington Biochemistry Inc.) using the BM Chemiluminescence kit (Roche Molecular Biochemicals) in accordance with the manufacturer’s protocol.

Lymphocyte Stimulation Analysis—The peripheral blood mononuclear cells were purified from heparinized peripheral blood obtained from apparently healthy adult donors after informed consent. The blood was centrifuged over Ficoll-Hypaque (Amersham Pharmacia Biotech,
Montreal, Quebec) density gradients and the peripheral blood mononuclear cells were collected as described (29). Cells were stimulated with concanavalin A (20 μg/ml; Sigma) in RPMI 1640 containing recombinant human IL-2 (100 units/ml; Cetus), 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units of penicillin and 100 μg of streptomycin/ml) as described (30). After incubation at 37°C in humidified 5% CO2 atmosphere for 3 days, the cells were collected for analysis.

Sialidase activity on the surface of lymphocytes was measured as described for COS-7 cells. To measure the total amount of the enzyme the assay was performed in the presence of 0.3% Triton X-100.

For immunoprecipitation 15 × 106 cells were placed on ice, washed twice with ice-cold PBS then lyzed by sonication in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) Nonident P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) supplemented with 5 μg/ml leupeptin, 50 mM sodium orthovanadate, 1 mM EDTA, 1.5 mM MgCl2, 50 mM NaF, complete protease inhibitory mixture (ICN, Costa Mesa, CA), and 1 mM α-toluenesulfonfonyl fluoride. The lysate was collected and centrifuged at 13,000 g for 10 min to remove the cell debris. 1.0 ml of lysate was incubated for 4 h with preimmune serum at a final dilution of 1/20. Then the pellet obtained from 300 μl of Pansorbin cells (Calbiochem) was added and the resulting suspension was incubated for 2 h at 4°C, followed by centrifugation for 10 min at 13,000 × g. Supernatants were incubated overnight with the antisialidase antibodies at a 1/100 final dilution, then for 2 h at 4°C with the pellet from 100 μl of Pansorbin cells and precipitated as above. The pellet was washed three times with 1 ml of RIPA buffer. The antigens were eluted from the pellet by the addition of 100 μl of a buffer containing 0.1 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M dithiothreitol, and 0.02% (w/v) bromphenol blue. The proteins were denatured by boiling for 5 min and 50 μl of each sample was subjected to SDS-PAGE according to Laemmli (27). After electrophoresis the proteins were electrotransferred from gels to nitrocellulose membrane. The sialidase detection was performed with rabbit antibodies and phosphotyrosine detection with monoclonal anti-phosphotyrosine Ab-1 antibodies (NeoMarkers, Fremont, CA) using the Vectastain ABC-Amp kit (Vector Laboratories, Burlingame, CA) in accordance with the manufacturer’s protocol.

For confocal immunomicroscopy cells were treated with LysoTracker Red, fixed, permeabilized, and blocked as described for COS-7 cells and fibroblasts. Cells were stained with rabbit anti-sialidase antibodies and/or monoclonal anti-human CTLA-4 (CD152) antibodies (PharMingen, Mississauga, Ontario). Cells were counterstained with Cy3-conjugated affinity purified anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), or Oregon Green 488-conjugated anti-rabbit IgG and Oregon Green 488-conjugated anti-mouse IgG antibodies.

**RESULTS**

The Distribution of Lysosomal Sialidase between Late Endosomal and Lysosomal Pools, and Its Pattern in AP3-deficient Cells Indicate That Sialidase Trafficking Resembles That of Lysosomal Membrane Proteins—A light mitochondrial fraction prepared from human liver homogenates was subjected to ultracentrifugation in a self-forming gradient of metrizamide (17). A typical distribution was obtained for the marker enzymes for lysosomes, microsomes, and mitochondria. As expected, lysosomal marker enzymes were found in two major peaks, previously described as “light” (fractions 3–5) and “dense” (fractions 11–13) lysosomes (Fig. 1). Previous data have shown that light lysosomes are identical to late endosomes, i.e. mannose 6-phosphate receptor-positive organelles containing internal lipid vesicles and responsible in most cells for the
Internalization of Lysosomal Sialidase

The C-terminal tetrapeptide YGTL has sequence similarities to the internalization signals of lysosomal membrane proteins and endocytosed surface receptors. The C-terminal tetrapeptide of sialidase, YGTL, resembles internalization signals previously identified in the cytoplasmic domains of several lysosomal membrane or endocytosed proteins. These include, for instance, the transferrin receptor, lysosomal acid phosphatase, LAMP-1, and CD63. Since the empirical algorithms for prediction of transmembrane domains in protein sequence did not provide a high score for such fragment in the C-terminal lobe of sialidase, we performed experiments aimed to detect directly if sialidase is an integral membrane protein. We performed extraction of COS-7 cells transfected with the wild-type sialidase and cathepsin A cDNA first with 50 mM acetic acid buffer (pH 5.2) and then with the same buffer, containing 1% of Zwitterionic detergent, 3A subunit of AP3 recognition of tyrosine-based signals on cargo proteins. The C-terminal fragment of sialidase we have covered in each fraction. We found (Fig. 3) that the majority of intracellular catabolism.

Soluble lysosomal enzymes targeted by a mannose 6-phosphate receptor-dependent pathway (i.e., β-galactosidase, N-acetyl β-hexosaminidase) were equally distributed between the light and dense lysosomes (Fig. 1). In contrast, lysosomal proteins, targeted to this organelle via clathrin-coated pits (i.e., LAMP-1, LAMP-2, β-glucosidase, and acid phosphatase) were present predominantly in the light pool of lysosomes (Fig. 1). Both measurement of sialidase activity (Fig. 1) and Western blotting of sialidase (not shown) demonstrated a distribution of sialidase between secondary endosomes and lysosomes similar to that of lysosomal membrane proteins which trafficking involves adaptor complexes.

Other indications of the trafficking pattern of sialidase was obtained from comparative studies involving LAMP-2, which moves from the TGN to the lysosome via clathrin-coated pits. We examined targeting of sialidase and LAMP-2, when the normal pathway from the TGN to the lysosome, was perturbed by deficiency of an AP3 adaptor complex, as occurs in HPS, type 2. In the two patients with this genetic disorder of vesicle formation, the gene coding for the β3A subunit of the heterotetrameric AP3 adaptor complex is mutated (16), and the patients' fibroblasts exhibit drastically reduced levels of AP3. Consequently, trafficking of lysosomal membrane proteins such as CD63, LAMP-1, and LAMP-2 to the plasma membrane is enhanced. This apparently results in a default mechanism when the normal TGN to lysosome trafficking, which requires AP3 recognition of tyrosine-based signals on cargo proteins destined for the lysosome, is impaired (16).

In any event, the AP3-deficient cells provide a model system in which LAMP-2 is misrouted to the plasma membrane, and in our experiments sialidase exhibited the same misrouting. In normal fibroblasts, both LAMP-2 and sialidase antibodies stained cytoplasmic perinuclear punctate structures which colocalized with lysosomal marker, LysoTracker Red dye (Fig. 2A, NORM). In contrast, in AP3-deficient cells (Fig. 2A, HPS-2) both antibodies showed mostly diffuse staining of the cell surface consistent with increased targeting of LAMP-2 and sialidase to the plasma membrane. In addition in AP3-deficient cells we detected the increased internalization of anti-sialidase antibodies (Fig. 2B) upon incubation of the cells with these antibodies for 15 min at 37°C. Previously similar results with AP3-deficient cells were obtained with antibodies to CD63, LAMP-1, and LAMP-2 (16). Along with the results of cellular fractionation experiments, these data suggest that sialidase and LAMP-2 use the same mechanism for lysosomal targeting.

Tyrosine. It is abbreviated Tyr-X-X-Φ, where Φ stands for bulky hydrophobic residue.

Sequence alignment with the internalization motifs of 6 proteins (cation independent mannose 6-phosphate receptor, transferrin receptor, lysosomal acid phosphatase, LAMP-1, LAMP-2, and CD63) indicates that the C-terminal fragment of human lysosomal sialidase contains a bulky hydrophobic amino acid (Leu32) two residues from the essential tyrosine (Tyr412). Therefore if Tyr312-Leu315 tail of sialidase would be exposed to the cytoplasm, it could represent the AP2 adaptor complex-binding motif.

Since the empirical algorithms for prediction of transmembrane domains in protein sequence did not provide a high score for such fragment in the C-terminal lobe of sialidase we have performed the experiments aimed to detect directly if sialidase is an integral membrane protein. We performed extraction of COS-7 cells transfected with the wild-type sialidase and cathepsin A cDNA first with 50 mM acetic acid buffer (pH 5.2) and then with the same buffer, containing 1% of Zwitterionic detergent 3–12 and assayed the percent of sialidase activity recovered in each fraction. We found (Fig. 3) that the majority (75–85%) of sialidase activity could not be extracted with buffer solution and required a detergent for solubilization.
Sialidase distribution between buffer extract (S1, Fig. 3), detergent extract (S2, Fig. 3), and unsoluble cell pellet (P2, Fig. 3) was similar to that of membrane lysosomal enzyme, β-glucosidase, whereas the majority of soluble lysosomal proteins such as cathepsin A, β-galactosidase (Fig. 3), or β-hexosaminidase (not shown) were recovered in the buffer extract.

Site-directed Mutagenesis of the Sialidase C Terminus Identifies the Tetrapeptide YGTL as an Internalization Signal—To determine whether the conserved Tyr412 and hydrophobic Leu415 are involved in targeting of sialidase to the endosome we prepared sialidase mutants with alanine codons replacing the wild-type codons for these amino acids. In another mutant, we replaced with alanine the Gly413 residue, presumably not important for endocytosis of sialidase.

The mutants, as well as wild-type sialidase, were expressed in COS-7 cells (20) together with human cathepsin A, which is necessary for expression of sialidase activity. The cell lysates were assayed for sialidase and cathepsin A activities 48 h after transfection (Fig. 4A). All transfected cells had similar cathepsin A activity suggesting the same transfection efficiency for all cells. Two of the expressed mutants, Y412A and L415A, had reduced sialidase activity, i.e. 30–45% of normal (Fig. 4A), while the activity of the G413A mutant was 50–70% of normal (Fig. 4A). In the cells transfected with wild-type or mutant sialidase, Western blotting of the expressed sialidase protein (Fig. 4B) revealed a 48–46-kDa band previously identified as mature, active sialidase (8).

The internalization of wild-type and mutant sialidase was studied by immunohistochemical staining with anti-sialidase antibody. The wild-type sialidase expressed in COS-7 cells was found in endosomal-lysosomal compartment; anti-sialidase immunofluorescence was observed in punctate structures co-localizing with the lysosomal markers LysoTracker Red (Fig. 5) or LAMP-2 (not shown). The G413A mutant showed similar localization, suggesting that conservation of Gly413 is not essential for internalization. In contrast, both Y412A and L415A mutants showed strong peripheral staining consistent with localization of the most of the expressed enzyme at the plasma membrane. In addition, diffuse cytoplasmatic staining, partially overlapping with LysoTracker Red and possibly representing the Golgi compartment, was apparent in some cells. Thus, the presence of both Tyr412 and Leu415 in the C-terminal peptide of sialidase appears necessary for internalization.

To quantify the distribution of sialidase mutants targeted to the endosomal/lysosomal compartment and to the cell surface, we assayed sialidase activity of purified lysosomal and plasma membranes. COS-7 cells harvested 48 h after transfection were divided into two portions. From one portion, lysosomal membranes were purified by subcellular fractionation and density centrifugation using a self-forming metrizamide gradient; from the other portion, plasma membranes were purified using poly-lysine-coated beads. The sialidase activity present on purified plasma membranes of COS-7 cells transfected with wild-type sialidase or the sialidase G413A mutant was very low (Fig. 6A, black bars). In contrast, a considerable amount of sialidase activity was found on plasma membranes purified from cells transfected with Y412A and L415A mutants (Fig. 6A, black bars). The activity of a control plasma membrane enzyme, alkaline phosphatase, was similar for nontransfected cells and for cells transfected with wild-type or mutant sialidase (Fig. 6A, open bars). The opposite effect was observed for lysosomal membranes. Transfection of COS-7 cells with wild-type sialidase or the sialidase G413A mutant increased the sialidase activity present on the lysosomal membranes ~3-fold (Fig. 6B, black bars). The sialidase activity on lysosomal membranes purified from cells transfected with the Y412A and L415A mutants was similar to that present on membranes purified from nontransfected control cells (Fig. 6B, black bars). The
activity of the endogenous lysosomal membrane enzyme, β-glucosidase, was similar for all the cells studied (Fig. 6B, open bars). The results suggest that the increased amount of sialidase present on the plasma membranes of the Y412A and L415A mutants resulted from impaired endocytosis.

A significant increase of sialidase activity on the surface of COS-7 cells transfected with Y412A and L415A mutants as compared with nontransfected cells was also observed when we incubated intact cells in an isotonic buffer containing sialidase substrate (Fig. 7A, black bars). A small increase of sialidase activity was also detected for cells transfected with the wild-type sialidase DNA or the G413A mutant. However, when the same assay was performed in the presence of 0.3% Triton X-100, which permeabilizes cell membranes (Fig. 7A, open bars), sialidase activity was reduced to the same level as that observed in nontransfected COS-7 cells (Control). Values represent mean ± S.D. of triplicate experiments.
bars), sialidase activity was 3-fold higher for the cells transfected with wild-type sialidase or the G413A mutant, reflecting the increased amount of enzyme localized within the cells. For the L415A and Y412A mutants, the activity was the same in the absence or presence of Triton, suggesting that all sialidase in the intact COS-7 cells transfected with L415A and Y412A mutants was available for the substrate. These results not only confirmed the findings observed for the purified membranes, but also showed that sialidase is located on the outside surface of the cell membrane.

**Fig. 7. Sialidase and cathepsin A activity on the cell surface of COS-7 cells transfected with sialidase mutants.** COS-7 cells transfected with cathepsin A (CathA) and wild-type (WT) or mutant sialidase cDNA were incubated in the isotonic buffer solution containing sialidase (A) or cathepsin A (B) substrates in the absence (black bars) or presence (open bars) of 0.3% (v/v) Triton X-100 as described. Specific sialidase and cathepsin A activities are shown as a fraction of that in COS-7 cells transfected with cathepsin A cDNA only (CathA). Values represent mean ± S.D. of triplicate experiments.

**Fig. 8. Immunohistochemical localization of sialidase and CTLA-4 in human lymphocytes.** Nonactivated (N) and activated (A) lymphocytes were probed with LysoTracker Red, fixed, stained with rabbit anti-sialidase antibodies or monoclonal anti-CTLA-4 antibodies, as indicated on the figure, and counterstained with Oregon Green 488-conjugated secondary antibodies. Alternatively, cells were double stained with anti-sialidase antibodies and anti-CTLA-4 antibodies and counterstained with Oregon Green 488-conjugated anti-mouse IgG antibodies and Cy3-conjugated anti-rabbit IgG antibodies. Slides were studied on an inverted confocal microscope. Magnification ×1000.

activated lymphocytes (9, 10) makes these cells particularly interesting for studying sialidase internalization. The availability of antisialidase antibodies allowed us to follow the intracellular distribution of sialidase using confocal immunofluorescence microscopy. In nonactivated cells, sialidase is localized within intracellular vesicles, which stain positively for the lysosomal marker LysoTracker Red (Fig. 8). Only weak expression of sialidase was observed on the cell surface. Upon stimulation with concanavalin A the fluorescence of the intracellular vesicles containing sialidase was reduced, whereas the cell periphery became brightly stained. This apparent translocation of sialidase from lysosomes to the cell surface was consistent with results of sialidase assays. Sialidase activity on the cell surface of intact cells incubated in an isotonic buffer containing substrate was ~4-fold higher for concanavalin A-activated lymphocytes (Fig. 9, black bars). Total sialidase activity assayed in the

**Sialidase Is Co-localized with CTLA-4 and Can Be Tyrosine-phosphorylated in Activated T Cells**—The previously described increase of neu-1-encoded sialidase activity on the surface of
presence of 0.3% Triton X-100 as described. Values represent mean ± S.D. of triplicate experiments.

![Image](http://www.jbc.org/content/55/3/3187/F9.large.jpg)

Fig. 9. Sialidase activity on the surface of control and concanavalin A-stimulated lymphocytes. Nonactivated (N) and activated (A) lymphocytes were incubated in the isotonic buffer solution containing sialidase substrate (SIAL), N-acetyl-β-hexosaminidase (HEX), or cathepsin A (CathA) substrates in the absence (black bars) or presence (open bars) of 0.3% (v/v) Triton X-100 as described. Values represent mean ± S.D. of triplicate experiments.

Similar re-distribution from lysosomes to the cell surface upon activation of T cells was reported for another protein, CTLA-4 antigen. This glycoprotein of the Ig superfAMILY functions as a co-receptor for CD28, but plays an opposite role by down-regulating T cell activation (42). Confocal immunomicroscopy studies of lymphocytes double stained with anti-CTLA-4 antibody and LysoTracker and with anti-CTLA-4 and anti-sialidase antibody showed that sialidase and CTLA-4 co-localize in the lysosomes of nonactivated cells and undergo translocation to the plasma membrane in activated lymphocytes (Fig. 8).

The intracellular distribution of CTLA-4 in lymphocytes is controlled by a complicated mechanism involving phosphorylation of the essential tyrosine of the internalization signal. In activated T cells, this phosphorylation blocks AP2-mediated endocytosis and retains CTLA-4 on the plasma membrane (43–46). To determine whether a similar type of regulation operates in the trafficking of sialidase we performed the immunoprecipitation of sialidase from the lysates of activated and nonactivated lymphocytes followed by the SDS-PAGE of the precipitated protein and Western blot using anti-sialidase and anti-phosphotyrosine antibodies. We observed that anti-phosphotyrosine antibodies reacted with sialidase (Fig. 10). The phosphorylation was significantly increased in activated compared with nonactivated cells. Although more experiments are needed to prove that Tyr\(^{422}\) is the particular amino acid residue, which undergoes phosphorylation, these results suggest that the interaction of sialidase and CTLA-4 with its adaptor complex is inhibited via the same mechanism.

DISCUSSION

Like other lysosomal proteins, sialidase is synthesized as a precursor which undergoes modification. After cleavage of a signal peptide and glycosylation, sialidase becomes a 48.3-kDa mature active enzyme found in the lysosome and present in the multienzyme lysosomal complex. However, the exact mechanism by which the sialidase precursor is sorted has remained unclear until now. Since sialidase contains three Asn-linked oligosaccharide side chains, it could be mannose 6-phosphorylated and targeted to the lysosome via mannose 6-phosphate receptors, as many soluble lysosomal enzymes. However, the mannose phosphorylation of sialidase is not sufficient for targeting the enzyme to the lysosomes (47). Based upon the intracellular distribution of human sialidase expressed in COS-1 cells transfected with sialidase cDNA alone or co-transfected with sialidase and human cathepsin A cDNA, van der Speel (47) suggested that the association of sialidase with cathepsin A occurs as early as in the endoplasmic reticulum and is required for proper sorting of the enzyme. In this scenario, sialidase would be transported to the lysosome via cathepsin A, which does obtain a robust mannose 6-phosphate recognition signal and binds to the mannose 6-phosphate receptor. In the absence of cathepsin A, sialidase is partially secreted and partially segregates to the endosomal compartment (47). This hypothesis is supported by data showing a significant increase of sialidase activity and immunoreactive material in lysosomes of cells co-transfected with sialidase and cathepsin A plasmids as compared with cells transfected with sialidase alone (48). However, this mechanism is not consistent with targeting of sialidase to the endosomal compartment observed in cultured fibroblasts of galactosialidosis patients lacking cathepsin A (8). In addition, sialidase was localized not only on the inner side of the lysosomal membrane and in the lysosomal lumen, but also on the plasma membrane and in small intracellular (possibly endocytic) vesicles (8). This suggests that sialidase may also be transported by a mechanism involving its targeting to the plasma membrane, followed by endocytosis.

In this study, we demonstrate a similarity in the distribution of sialidase and lysosomal membrane proteins that are targeted to the lysosome by adaptor complexes AP2 (acid phosphatase) or AP3 (LAMP-2). Similarly to lysosomal membrane enzyme, β-glucosidase, the majority of sialidase expressed in COS-7 cells could not be extracted with buffer solution, but required high (1%) concentration of detergent for solubilization (Fig. 3). We also found that both LAMP-2 and sialidase exhibit the same misrouting in AP3-deficient (HPS-2) cells, in which trafficking of lysosomal membrane proteins to the plasma membrane is enhanced. Both anti-sialidase and anti-LAMP-2 antibodies showed a diffuse staining in AP3-deficient cells instead of cytoplasmic perinuclear punctate structures detected.
in normal cells. Although these results do not prove that sialidase lacks internalization in AP3-deficient cells they suggest that sialidase and LAMP-2 use the same mechanism for lysosomal targeting.

We have demonstrated that the C-terminal tetrapeptide of sialidase 412YGTI,415 represents the tyrosine-containing lysosomal targeting signal, and that Tyr412 and Leu415 amino acid residues are essential for rapid endocytosis of sialidase. Specifically, the sialidase substitution mutants Y412A and L415A were sorted to the plasma membrane but not internalized. Altogether our data suggest that lysosomal sialidase contains the internalization signal most probably capable of binding both AP2 and AP3 adaptor complexes.

The identified mechanism of sialidase targeting not only provides essential information about the enzyme's biogenesis but also extends our knowledge about several important physiological processes involving this enzyme. Several studies have shown that neu-1-encoded sialidase, in addition to its role in intralysosomal catabolism of sialylated glycoconjugates, is also involved in cellular signaling during the immune response. In particular, T cells require sialidase for both early production of IL-4 and the IL-4 priming of conventional T cells to become active IL-4 producers (49, 50). During the activation of T cells, sialidase is expressed on the plasma membrane where it participates in desialylation of surface antigen-presenting molecules such as myosin heavy chain class I, required to render T cells responsive to antigen presenting cells (51), and GMI-ganglioside, which modulates Ca2+ immobilization and regulates IL-4 production (50). In addition, sialidase of T lymphocytes converts the group specific component or Gc protein into an internalization signal most probably capable of binding both AP2 and AP3 adapter complexes.

Acknowledgments—We thank Dr. Mila Ashmarina for stimulating discussions, Dr. Mogens Thomsen for critical reading of the manuscript, and Lilianne Gallant for help in preparation of the manuscript.

REFERENCES
1. d’Azzo, A., Hoogeveen, A., Reuser, A. J., Robinson, D., and Galjaard, H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4535–4539
2. van der Horst, G. T., Galjaard, H., van der Horst, J., d’Azzo, A., Galjaard, H., and Verheijen, F. W. (1989) J. Biol. Chem. 264, 1317–1322
3. Pshezhetsky, A. V., and Potier, M. (1996) J. Biol. Chem. 271, 28359–28365
4. d’Azzo, A., Andria, G., Strisciuglio, P., and Galjaard, H. (2001) Metabolic and Molecular Bases of Inherited Disease, Vol. 5, pp. 3811–3826, McGraw-Hill, New York
5. Thomas, G. H. (2001) The Metabolic and Molecular Bases of Inherited Disease, Vol. 3, pp. 3507–3534, McGraw-Hill, New York
6. Miyagi, T., Hata, K., Hasegawa, A., and Aoyagi, T. (1993) Glycocon. J. 10, 45–49
7. Verheijen, W. F., Panne, J. H. C., van Diggelen, O. P., Bakker, H. D., Loonen, M. C., Durand, P., and Galjaard, H. (1983) Biochem. Biophys. Res. Commun. 117, 470–478
8. Vinogradov, M. V., Michaud, L., Zenetssev, A. V., Lukong, K. E., El-Alfy, M., Morales, C. R., Potier, M., and Pshezhetsky, A. V. (1998) Biochem. J. 336, 641–650
9. Landolfi, N. F., Leone, J., Womack, J. E., and Cook, R. G. (1985) Immunogenetics 22, 159–167
10. Naraparaju, V. R., and Yamamoto, N. (1994) Immunol. Lett. 43, 143–148
11. Carrillo, M. B., Milner, C. N., Ball, S. T., Snock, M., and Campbell, R. D. (1997) Glycobiology 7, 975–986
12. Hirst, J., and Robinson, M. S. (1998) Biochim. Biophys. Acta 1403, 173–193
13. Peters, C., and von Figura, K. (1994) FEBS Lett. 346, 108–114
14. Peare, B. M., Smith, C. J., and Owen, D. J. (2000) Curr. Opin. Struct. Biol. 10, 220–229
15. Jarussee, N., and Kelly, R. B. (2000) Traffic 1, 378–384
16. Dell’Angelica, E. C., Shotelersuk, V., Aguilar, R. C., Gahl, W. A., and Bonifacino, J. S. (1999) Mol. Cells 3, 11–21
17. Wattiaux, R., and Watia-Sloof, A. (1983) Iodinated Density Gradi- ent Media: A Practical Approach, Oxford University Press, Oxford
18. Ling, M. M., and Robinson, B. H. (1997) Anal. Biochem. 254, 157–178
19. Pshezhetsky, A. V., Richard, C., Michaud, L., Igoufara, S., Wang, S., Eldiger, M. A., Qu, J., Leclere, D., Gravel, R., Dallaire, L., and Potier, M. (1997) Nat. Genet. 15, 316–320
20. Lukong, K. E., Eldiger, M. A., Chang, Y., Richard, C., Thomas, G., Carey, W., Tylik-Szymanska, A., Czartoryski, B., Buchholz, T., Criado, G., Palmeri, S., and Pshezhetsky, A. V. (2000) Hum. Mol. Genet. 9, 1075–1085
21. Potier, M., Mameli, L., Belisle, M., Dallaire, L., and Melancon, S. B. (1979) J. Biol. Chem. 254, 28359–28365
22. Rome, L. H., Garvin, A. J., Alietta, M. M., and Neufeld, E. F. (1979) Cell 17, 143–153
23. Oda, S., and O’Brien, J. S. (1968) Science 160, 1002–1004
24. Seymour, C. A., and Peters, T. J. (1977) Clin. Sci. Mol. Med. 52, 229–239
25. Gingras, R., Richard, C., El-Alfy, M., Morales, C. R., Potier, M., and Pshezhetsky, A. V. (1999) J. Biol. Chem. 274, 11742–11750
26. Kalisch, D. I., and Cohen, C. M. (1983) J. Biol. Chem. 258, 141–146
27. Kominami, E., Fujisawa, T., and Saito, T. (2000) J. Biol. Chem. 275, 27158–27164
28. Ahmad, A., Sharif-Afshari, E., Fawaz, L., and Menezes, J. (2000) J. Virol. 74, 7196–7203
29. Iida, T., Ohno, H., Nakaseko, C., Sakuma, M., Takeda-Ezaki, M., Arase, H., Komianini, E., Fujisawa, T., and Saito, T. (2000) J. Immunol. 165, 5062–5068
30. Guarnieri, F. G., Artberrion, L. M., Pennzo, M. B., Cha, Y., and August, J. T. (1993) J. Biol. Chem. 268, 14144–14146
31. Akasaka, K., Michihara, A., Fukuzawa, M., Kinosita, H., and Tsuji, H. (1994) J. Biochem. (Tokyo) 116, 670–676
32. Prince, L. S., Peter, K., Hatton, S. R., Zaluzianski, L., Cotlin, L. F., Clancy,
34. Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S. Q., Trowbridge, I. S., and Tainer, J. A. (1990) Cell 63, 1061–1072
35. Collawn, J. F., Lai, A., Domingo, D., Fitch, M., Hatton, S., and Trowbridge, I. S. (1993) J. Biol. Chem. 268, 21686–21692
36. Ozaki, K., Itoh, N., and Kawasaki, T. (1993) J. Biochem. (Tokyo) 113, 271–276
37. Okamoto, C. T., Shia, S. P., Bird, C., and Mostov, K. E. (1992) J. Biol. Chem. 267, 39925–39932
38. Canfield, W. M., Johnson, K. F., Ye, R. D., Gregory, W., and Kornfeld, S. (1991) J. Biol. Chem. 266, 5682–5688
39. Jadot, M., Canfield, W. M., Gregory, W., and Kornfeld, S. (1992) J. Biol. Chem. 267, 11069–11077
40. Lehmann, L. E., Eberle, W., Krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K., and Peters, C. (1992) EMBO J. 11, 4391–4399
41. Gottschalk, S., Waheed, A., Schmidt, B., Laidler, P., and von Figura, K. (1989) EMBO J. 8, 3215–3219
42. Ostrov, D. A., Shi, W., Schwartz, J. C., Almo, S. C., and Nathenson, S. G. (2000) Science 290, 816–819
43. Leung, H. T., Bradshaw, J., Cleaveland, J. S., and Linsley, P. S. (1995) J. Biol. Chem. 270, 25107–25114
44. Bradshaw, J. D., Lu, P., Leytze, G., Rodgers, J., Schieven, G. L., Bennett, K. L., Linsley, P. S., and Kurtz, S. E. (1997) Biochemistry 36, 15875–15882
45. Miyatake, S., Nakaseko, C., Umemori, H., Yamamoto, T., and Saio, T. (1998) Biochem. Biophys. Res. Commun. 19, 444–448
46. Chikuma, S., Murakami, M., Tanaka, K., and Uede, T. (2000) J. Cell. Biochem. 78, 241–250
47. van der Spoel, A., Bonten, E., and Azzo, A. (1998) EMBO J. 17, 1588–1597
48. Bonten, E. J., van der Spoel, A., Fornerod, M., Grøsveld, G., and d’Azze, A. (1998) Genes Dev. 10, 3156–3168
49. Chen, X. P., Enioutina, E. Y., and Daynes, R. A. (1997) J. Immunol. 158, 3070–3080
50. Chen, X. P., Ding, X., and Daynes, R. A. (2000) Cytokine 12, 972–985
51. Landolfi, N. F., and Cook, R. G. (1986) Mol. Immunol. 23, 297–309
52. Yamamoto, N., and Kumahiro, R. (1993) J. Immunol. 151, 2794–2802
53. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnoffsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Cheu, M. M., Hanafusa, H., Schaffhausen, B. G., and Cantley, L. C. (1993) Cell 72, 767–778
54. Hinek, A. (1996) Biol. Chem. 377, 471–480
Intracellular Distribution of Lysosomal Sialidase Is Controlled by the Internalization Signal in Its Cytoplasmic Tail

Kiven E. Lukong, Volkan Seyrantepe, Karine Landry, Stéphanie Trudel, Ali Ahmad, William A. Gahl, Stéphane Lefrançois, Carlos R. Morales and Alexey V. Pshezhetsky

J. Biol. Chem. 2001, 276:46172-46181.
doi: 10.1074/jbc.M104547200 originally published online September 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104547200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 20 of which can be accessed free at http://www.jbc.org/content/276/49/46172.full.html#ref-list-1