Lysosomal Sialidase (Neuraminidase-1) Is Targeted to the Cell Surface in a Multiprotein Complex That Facilitates Elastic Fiber Assembly*

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We have established previously that the 67-kDa elastin-binding protein (EBP), identical to the spliced variant of β-galactosidase, acts as a recyclable chaperone that facilitates secretion of tropoelastin. (Hinek, A., Keeley, F. W., and Callahan, J. W. (1995) Exp. Cell Res. 220, 312–324). We now demonstrate that EBP also forms a cell surface-targeted molecular complex with protective protein/cathepsin A and sialidase (neuraminidase-1), and provide evidence that this sialidase activity is a prerequisite for the subsequent release of tropoelastin. We found that treatment with sialidase inhibitors repressed assembly of elastic fibers in cultures of human skin fibroblasts, aortic smooth muscle cells, and ear cartilage chondrocytes and caused impaired elastogenesis in developing chick embryos. Fibroblasts derived from patients with congenital sialidosis (primary deficiency of neuraminidase-1) and galactosialidosis (secondary deficiency of neuraminidase-1) demonstrated impaired elastogenesis, which could be reversed after their transaction with neuraminidase-1 cDNA or after treatment with bacterial sialidase, which has a similar substrate specificity to human neuraminidase-1. We postulate that neuraminidase-1 catalyzes removal of the terminal sialic acids from carbohydrate chains of microfibrillar glycoproteins and other adjacent matrix glycoconjugates, unmasking their penultimate galactosugars. In turn, the exposed galactosugars interact with the galectin domain of EBP, thereby inducing the release of transported tropoelastin molecules and facilitating their subsequent assembly into elastic fibers.

Mature elastic fibers and laminae present in the extracellular matrix of connective tissues of different organs and blood vessel walls are complex structures made of polymeric (insoluble) elastin in which polypeptide chains of tropoelastin are covalently cross-linked and assembled on a scaffold of 12-nm microfibrils consisting of several glycoproteins, e.g. fibrillins and microfibril-associated glycoproteins (1–11). Molecules of monomeric 70-kDa tropoelastin are synthesized by fibroblasts, chondrocytes, and smooth muscle cells and are secreted and properly aligned with one another for the subsequent lysyl oxidase-mediated cross-linking (12). The mechanisms governing tropoelastin secretion and assembly are not fully elucidated. The current data indicate that highly hydrophobic and non-glycosylated tropoelastin associates with several intracellular proteins that chaperone it through the intracellular compartments. Davis et al. (13) documented that, in the ergastoplasmic reticulum, tropoelastin binds to BiP and the peptidylprolyl cis-trans isomerase FKBP65. Our immunohistochemical and biochemical studies have indicated that tropoelastin present in endosomal and Golgi compartments is escorted by the 67-kDa elastin-binding protein (EBP)2 (14, 15), which protects tropoelastin from premature intracellular self-aggregation and an association with serine proteinases (16, 17). We have established that, after delivering tropoelastin to the growing elastic fibers, the EBP molecules dissociate from tropoelastin, return to endocytic compartments (but not to the lysosomes), bind again to newly synthesized tropoelastin in the trans-Golgi network, and recycle back to the cell surface (18). Of particular importance is the observation that 67-kDa EBP also has a separate galectin domain and that binding of galactosugars to this site induces conformational changes in the EBP molecule, resulting in its dissociation from tropoelastin (14, 15, 19–21). This leads to the paradigm that the release of newly secreted tropoelastin molecules from their EBP transporters is highly coordinated and occurs on the cell surface following the interaction between EBP and galactosugar moieties, presumably those protruding from carbohydrate chains of glycoproteins forming the microfibrillar scaffold of new elastic fibers (22, 23). We have also established that such coordinated assembly of tropoelastin into elastic fibers can be disrupted by pericellular accumulation of galactosugar-bearing moieties such as chondroitin sulfate and dermatan sulfate, which induce premature shedding of EBP from the cell surface and release of tropoelastin far away from microfibrillar acceptors (14–23, 25, 26, 57, 58).

During our initial studies that led to the identification of 67-kDa EBP, we found that several other proteins of unknown identity (61, 55, 45, and 32) can be co-purified with EBP from the detergent extract of plasma membrane fractions on elastin affinity columns despite the fact that they do not bind elastin (14, 15). This suggested that 67-kDa EBP is engaged in a multiprotein complex. Because our previous studies definitively established that 67-kDa EBP is identical to the enzymatically inactive spliced variant of β-galactosidase (S-gal) (24–26), we speculated that other proteins isolated in association with 67-kDa EBP could...
be similar or identical to lysosomal sialidase (neuraminidase-1 (Neu1); EC 3.2.1.18) and to protective protein/cathepsin A (PPCA; EC 3.4.16.1), which normally form a molecular complex with the lysosomally targeted active β-galactosidase (EC 3.2.1.23) (27). This concept has been confirmed by us (22, 23, 28) and others (29–31).

The results described in this study demonstrate further that proteins associated with S-gal/EBP in the cell surface-targeted multiprotein complex can be immunocharacterized as Neu1 and PPCA. We also show that fibroblasts derived from patients with congenital sialidosis (primary deficiency of neuraminidase-1) and galactosialidosis (secondary deficiency of neuraminidase-1) demonstrate impaired elastogenesis, which can be reversed after their transduction with Neu1/PPCA cDNA or after treatment with bacterial sialidase with similar substrate specificity compared with human Neu1. This and other results presented in this study indicate that the enzymatic activity of cell surface-targeted Neu1 is a prerequisite for the release of transported tropoelastin molecules from their S-gal/EBP chaperone and for their subsequent assembly into extracellular elastic fibers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Medium 199, phosphate-buffered saline, fetal bovine serum, and other tissue culture reagents were obtained from Invitrogen. Neuraminidase (Clostridium perfringens Type V), the neuraminidase inhibitor 2,3-dehydro–2-deoxy–N-acetylneuraminic acid (ddNeuAc), enzyme substrates, and all reagent-grade chemicals were purchased from Sigma. Two other preparations of neuraminidase inhibitors, 2,3-didehydro–2-deoxy–N-acetylneuraminic acid (Neu5Ac2en) and 2,3-didehydro–2,4-dideoxy–4-guanidinyl–N-acetylneuraminic acid (dgNeu5Ac2en), were prepared as described previously (32–35).

Anti-tropoelastin polyclonal antibody (36) was obtained from Elastin Products Co., Inc. (Owensville, MO). Monoclonal antibody to fibronectin and polyclonal antibodies to collagen type I and fibrillin-1 were purchased from Chemicon International Inc. (Temecula, CA). Polyclonal anti-S-gal antibody recognizing EBP was raised against synthetic peptides with the amino acid sequence of the elastin-binding domain of S-gal (25), and monoclonal antibody BCZ-67 recognizing human EBP was raised against antigen from the bovine ciliary zone (37). Rabbit polyclonal antibody specific to human Neu1 and not cross-reacting with other human sialidases (46) and chicken antibody to human lysosomal PPCA (a generous gift from Dr. J. W. Callahan, Division of Structural Biology and Biochemistry, The Hospital for Sick Children, Toronto) (38) were also used. Fluorescein-labeled goat anti-rabbit and rhodamine-labeled goat anti-mouse secondary immunoglobulin F(ab’)2 fragments, nuclear stains, propidium iodide, and 4”,6-diamidino-2-phenylindole were purchased from Sigma. The enhanced chemiluminescence Western blotting detection kit (ECL), [3H]leucine, and [3H]valine were obtained from Amersham Biosciences. Species- and type-specific secondary antibodies conjugated to gold particles for electron microscopy immunolocalization were obtained from Janssen Life Science Products (Piscataway, NJ). Horseradish peroxidase-conjugated secondary antibodies used in Western immunoblotting were supplied by Roche Applied Science. Rhodamine-conjugated Limax flavus agglutinin (LFA), which specifically binds to sialic acid residues (39), and fluorescein-conjugated galactosugar-specific (Galβ1–3GalNAc) peanut agglutinin (PNA) (40) were purchased from EY Laboratories, Inc. (San Mateo, CA).

**Cell Cultures**—Aortic smooth muscle cells (ASMCs) were propagated from small aortic fragments obtained during the autopsy of a patient who died of a road accident. Elastic cartilage fragments (source of chondrocytes) were obtained from a 1-year-old patient at the time of surgery for extirpation of pre-auricular tags. Because a functional deficiency of Neu1 occurs in two genetically distinct diseases (sialidosis, caused by primary lesions in the NEU1 gene (41–44), and galactosialidosis, in which a combined secondary deficiency of Neu1 and β-galactosidase occurs as a result of a primary deficiency of PPCA (45–48), we also tested cells derived from patients with these diseases. Skin biopsies from three patients diagnosed with congenital sialidosis (patients 4028, 4029, and 4079) and three patients diagnosed with galactosialidosis (patients 5974, 5975, and 5976) were used. Additionally, skin fibroblasts derived from two patients (patients 4032 and 8982) with GM1 gangliosidosis, bearing a nonsense mutation of the β-galactosidase gene (25,
26), and biopsies from three normal children (patients 4212, 3858, and 4992) of matching ages were harvested and used as the primary source of fibroblasts for our studies. All cells were originally isolated by collagenase digestion of the initial tissues and then passaged two to five times by trypsinization and maintained in MEM-supplemented with 20 mM HEPES, 1% antibiotics/antimycotics, and 10% fetal bovine serum. Cultured fibroblasts were used for isolation and characterization of the EBP complex. Cultured fibroblasts, chondrocytes, and ASMCs were maintained in the presence or absence of sialidase inhibitors (ddNeuAc, Neu5Ac2en, and dgNeu5Ac2en) at 100–250 μM, blocking antibody to human Neu1 at 2 μg/ml, and exogenous sialidase from C. perfringens at 20–100 milliunits/ml. This bacterial sialidase shares substrate specificity with Neu1 and displays a high degree of sequence homology to this human enzyme (49, 50). The effects of these reagents on the production of elastin and other extracellular matrix (ECM) components (fibrillin-1, collagen type I, and fibronectin) was assessed in 7-day cell cultures by immunofluorescence microscopy, immunogold electron microscopy, and biochemical and morphometric evaluations.

Affinity Chromatography on Insoluble Elastin and Western Blotting—
Cultured fibroblasts (10 × 10^6 cells) were harvested by scraping. Plasma membranes were prepared from a low speed pellet (400 × g) by flotation in isotonic Percoll solution, followed by a hypotonic wash and flotation in a discontinuous sucrose gradient as described previously (51). This plasma membrane fraction has been positively identified by the presence of marker enzymatic activity (alkaline phosphodiesterase, EC 3.1.4.1) detected at pH 9 with sodium thymidine 5-monophosphate p-nitrophenyl ester substrate (51). It also negatively tested for lysosomal enzyme β-hexosaminidase A activity (52). Extracts of whole cell pellets and extracts of plasma membrane fractions were prepared from parallel cultures (53) and chromatographed on insoluble elastin as described previously (25). The elastin affinity slurries were washed with 0.1 M HEPES and then suspended in 2 μg/ml, blocking antibody to human Neu1 at 2 μg/ml, and exogenous sialidase from C. perfringens at 20–100 milliunits/ml. This bacterial sialidase shares substrate specificity with Neu1 and displays a high degree of sequence homology to this human enzyme (49, 50). The effects of these reagents on the production of elastin and other extracellular matrix (ECM) components (fibrillin-1, collagen type I, and fibronectin) was assessed in 7-day cell cultures by immunofluorescence microscopy, immunogold electron microscopy, and biochemical and morphometric evaluations.

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FIGURE 2. A, immunofluorescence microscopy of permeabilized ASMCs demonstrated that polyclonal antibody to human lysosomal Neu1 localized to intracellular lysosomes and to the cell surface of ASMCs. Magnification ×400. B, the two confocal micrographs show optical sections of cultured ASMCs that had been exposed to sodium azide, cooled to 15 °C (to avoid endocytosis), and then externally labeled with fluorescein-conjugated anti-Neu1 polyclonal antibody. The first optical section (left) demonstrates localization of Neu1 to the cell surface. The second optical section (right), which was taken at a 5-μm depth, demonstrates the “empty” cell interior and decoration of the cell membrane with anti-Neu1 antibody. Magnification is ×600. C, confocal microscopy demonstrated that polyclonal antibody to lysosomal Neu1 and monoclonal antibody raised against EBP co-localized on the surface of non-fixed ASMCs. Magnification is ×1000.
saline and tested for the enzymatic activities of sialidase, β-galactosidase, and β-hexosaminidase A.

**Enzyme Assays**—Aliquots of whole cell and plasma membrane extracts, elastin slurries containing bound proteins, phosphate-buffered saline washes, and lactose eluates from elastin slurries were mixed with the appropriate substrate solutions in enzymatic activity assays. To test neuraminidase activity, 4-methylumbelliferyl-β-N-acetyl-α-D-neuramic acid was used as a substrate (54, 55). The activities of β-galactosidase and β-hexosaminidase A were also assayed as described previously using 4-methylumbelliferyl glycosides as substrates (56). Protein determination was performed with a Bio-Rad protein assay kit.

**Immunocytochemistry**—Subconfluent cultures of human ASMCs were fixed and permeabilized in 100% cold methanol, immunostained, and evaluated by immunofluorescence microscopy. EBP was localized with either polyclonal anti-S-gal antibody (25) or monoclonal antibody BCZ-67 (37). Two other components of the cell surface-targeted elastin-binding complex were localized with the polyclonal antibodies raised against human Neu1 (46) and human PPCA (38). Antibody to β-hexosaminidase A has also been used to localize lysosomes (52). The co-localization of EBP with Neu1 or PPCA was performed by simultaneous incubation of cultured cells with the respective pairs of antibodies (monoclonal antibody BCZ-67 and anti-Neu1 polyclonal antibody; rabbit anti-S-gal and chicken anti-PPCA antibodies). The nuclei were counterstained with 4',6-diamidino-2-phenylindole. In parallel experiments, the monolayer culture of ASMCs that were cooled to 15 °C in the presence of sodium azide was externally labeled with fluorescein-conjugated anti-Neu1 polyclonal antibody as described previously (18). Similarly, prepared ASMCs were also externally labeled with fluorescein-conjugated anti-Neu1 polyclonal antibody and rhodamine-conjugated anti-EBP monoclonal antibody and evaluated by confocal microscopy.

To visualize the effects of sialidase inhibitors or bacterial sialidase on deposition of elastin and other ECM components by human ASMCs, ear chondrocytes, and skin fibroblasts, 7-day confluent cultures treated with the above-mentioned reagents were fixed in 100% cold methanol. The deposition of ECM components was then detected with polyclonal antibodies to tropoelastin, fibrillin-1, and collagen type I or with monoclonal antibody to human fibronectin as described previously (57, 58). The immunoreactions were visualized with the appropriate fluorescein-conjugated secondary antibodies. The nuclei were counterstained with propidium iodide.

Immunodetection of Neu1, PPCA, and EBP and their mutual subcellular co-localization, as well as production of elastic fibers by cells cultured in the presence and absence of the sialidase inhibitor ddNeuAc and exogenous bacterial sialidase, were confirmed by a immunogold technique and electron microscopy using a post-embedding method as described previously (20). In all immunostaining procedures, controls included substitution of non-immune and antigen-adsorbed sera for the primary antibody.

To test whether galactosugars and sialic acid residues can co-localize with the elastic fiber microfibrils, we used rhodamine-conjugated LFA, which specifically binds to sialic acid residues, and fluorescein-conjugated PNA, which specifically binds to galactosugars (Galβ1–3GalNAc residues) (39, 40). The lectins were used separately, in double staining, and in combination with polyclonal antibody recognizing fibrillin-1 (detected with secondary antibody conjugated to either fluorescein or rhodamine). All above-described specimens were then mounted in Eilanol and examined under a Nikon Eclipse E1000 microscope equipped with a source of fluorescent light and a set of multiple filters. The images were obtained with a cooled CCD camera (Retiga EX, QImaging Corp.). Morphometric analysis of extracellular matrix components was then performed using Image-Pro Plus software (Media Cybernetics, Inc., Silver Springs, MD) as described previously (57, 58).

**Expression of Neu1 cDNA in Sialidosis Fibroblasts**—Full-length human Neu1 cDNA and human PPCA cDNA cloned into the pCMV expression vector were obtained as described previously (59). Skin fibroblasts from normal controls and from a sialidosis type II patient (line WG0544), characterized by very low sialidase mRNA levels and activity (59), were cultured in 60-mm dishes and on coverslips in Eagle's minimal essential medium (Mediatech, Inc., Washington, D. C.) supplemented with 10% (v/v) fetal calf serum (MultiCell Technologies) and antibiotics. At 70% confluence, cells were transduced with pCMV-sialidase and pCMV-cathepsin A expression vectors using Lipofectamine Plus reagent (Invitrogen) following the manufacturer's protocol (60). The transduction resulted in an ~6-fold increase in specific sialidase activity in normal cells and an ~150-fold increase in specific sialidase activity in sialidosis cells measured 48 h after transfection as described (54). In both cells, specific cathepsin A activity (61) was increased 2.5–3-fold. The transfection efficiency detected immunohistochemically with anti-Neu1 antibody was between 8 and 10%.

**Measurement of Soluble Elastin (Tropoelastin)**—Quadruplicate cultures of human ASMCs, elastic ear cartilage chondrocytes, and skin fibroblasts (plated at 2 × 10^6 cells/dish) were grown to confluency and then maintained for 7 days in the presence or absence of *C. perfringens* sialidase or the sialidase inhibitor ddNeuAc at the concentrations spec-
ified in the figure legends. All cultures were incubated for the last 24 h with \[^{3}H\]valine. At the end of the experiments, the conditioned medium was collected, and cell layers were extracted for 1 h at 4°C with 0.5 M acetic acid in the presence of a proteinase inhibitor mixture. Both the conditioned medium and neutralized cell extracts were then separately immunoprecipitated with monospecific antibody to bovine tropoelastin as described previously (57, 58). Newly synthesized tropoelastin (from cell extracts) and secreted tropoelastin (from the medium) were assessed quantitatively by scintillation counting of solubilized protein A-Sepharose pellets containing the immunoprecipitated proteins. The radioactivity present in each sample of the cell extract and conditioned medium was matched with the particular culture and expressed as cpm/µg of DNA. The means ± S.D. were calculated from three separate experiments.

Measurement of Insoluble Elastin Produced by Cultured Cells—Deposition of insoluble elastin was reflected by the levels of radioactive valine present in residues remaining after boiling the cell layers of the same cultures in 0.1 N NaOH for 45 min (55, 56). This procedure removes all cellular and extracellular components except the cross-linked elastin. The results were expressed as cpm and normalized per DNA content determined in the soluble fractions of NaOH extracts.

Measurement of Total Protein Synthesis—Total protein synthesis in parallel cultures maintained for 24 h with and without exogenous sialidase and sialidase inhibitors was also determined in triplicate by radio-
RESULTS

The 67-kDa Spliced Variant of β-Galactosidase That Binds Elastin Also Forms a Cell Membrane-targeted Complex with Neu1 and PPCA—The elastin-binding complex isolated from the cell membrane fraction of normal human fibroblasts by elastin affinity chromatography contained multiple Coomassie Blue-detectable proteins: the 67-kDa protein reacted with anti-S-gal antibody; the 61- and 46-kDa species were reactive with antibody raised against human Neu1; and the 55-, 32-, and 20-kDa bands were immunoreactive with antibody to human PPCA (Fig. 1A). Additional blotting with 125I-labeled tropoelastin, followed by autoradiography, demonstrated than only the 67-kDa component of this molecular complex bound radioactive tropoelastin (Fig. 1A). The results of assays using specific artificial substrates showed that 10–12% of the total sialidase activity present in the whole cell extract of human fibroblasts bound to elastin affinity columns and could be eluted from them with lactose. The elastin slurrers also bound ~70% of the sialidase activity detected in the cell membrane extract. Much of this activity could then be extracted from elastin slurrers with lactose. The elastin affinity slurrers did not retain β-galactosidase or β-hexosaminidase A activities (Table 1).

![Image](234x26 to 262x38)

The 67-kDa variant of β-galactosidase binds to elastin but only when Neu1 is present. A soluble variant of Neu1 eluted from elastin slurrers. In contrast, a cell membrane extract of thyroid cells (Fig. 1D) bound to elastin slurrers and could only be eluted with Neu1-specific antibody, suggesting that Neu1 is an essential component of this complex.

![Image](313x400 to 564x733)

**FIGURE 5.** Representative micrographs of histological sections of lungs and aortas from full-term chicken embryos treated in the presence and absence of the neuraminidase inhibitor 4-deoxy-4-guanidino-Neu5Ac2en. In contrast to untreated embryos that have abundant elastic fibers surrounding the airways (A and C), lungs from chicken embryos that received the neuraminidase inhibitor showed dilated parabronchi (B) and distended air capillaries (D) surrounded by fragmented and diffused elastic fibers. In contrast to the aorta from a solvent-treated chicken embryo with compact elastic lamellae (E), the aorta from a sialidase inhibitor-treated embryo showed an impaired architecture of elastic lamellae (F). Staining was performed with Hart’s elastic stain counterstained with tartarazine. Magnification is ×50.

The fluorescent immunostaining with anti-Neu1 antibody performed on fixed and permeabilized cells demonstrated immunolocalization of Neu1 in lysosomes (big vesicles) and in the cell membrane (small dots) (Fig. 2A). The parallel cultures immunostained with antibody to the lysosomal enzyme β-hexosaminidase A localized this epitope to the big vesicles only (data not shown). The immunolocalization of Neu1 to the cell surface was also confirmed by confocal microscopy of the externally labeled, unfixed, and non-permeabilized cells (Fig. 2B) and by immunogold electron microscopy (Fig. 3A). The immunogold technique also confirmed the cell surface localization of EBP (Fig. 3B) and PPCA (Fig. 3C). Both confocal microscopy (Fig. 2C) and immunogold electron microscopy (Fig. 3D) allowed for the cell-surface co-localization of Neu1 and EBP. Taken together, these findings and the additional intracellular and cell-surface co-localization of Neu1 and tropoelastin (Fig. 1B) validated the predicted identity of all subunits of the cell surface-targeted elastin-binding complex and encouraged experiments testing a putative function of cell-surface Neu1 in elastogenesis.

**Inhibition of Endogenous Sialidase Activity Disrupts Normal Elastogenesis**—Metabolic labeling of confluent cultures of human ASMCs with [3H]valine, followed by extraction of newly deposited insoluble elastin from 7-day cultures, indicated that these cells significantly decreased their deposition of cross-linked elastin (a major component of mature elastic fibers) when cultured in the presence of sialidase inhibitors or in the presence of anti-Neu1 IgG antibody (Fig. 4A). At the same time, cultures treated with these reagents demonstrated even higher levels of newly synthesized, but not assembled, immunoprecipitable (soluble) tropoelastin compared with untreated controls. Treatment with sialidase inhibitors did not affect total protein synthesis. Immunostaining of 7-day cultures of ASMCs with anti-elastin polyclonal antibody demonstrated that treatment of these cells with ddNeuAc (added at days 1, 3, and 5 during the course of 7-day cultures) induced a dose-dependent inhibition of elastic fiber formation (Fig. 4B).

The requirement of sialidase activity for proper elastic fiber assembly was further confirmed by the results of in vivo experiments. Histological analysis of full-term chicken embryos treated with different sialidase inhibitors indicated that each inhibitor induced very similar effects, resulting in a significant inhibition of elastogenesis or elastin assembly in lungs and aortas. The representative micrographs show that, in contrast to untreated embryos, which have abundant elastic membranes surrounding the airways (Fig. 5, A and C), lungs from chicks treated with the neuraminidase inhibitor 4-deoxy-4-guanidino-Neu5Ac2en demonstrated dilated parabronchi with shallow and poorly defined crypts surrounded by sparse, thin elastic fibers (Fig. 5B). Longitudinal sections through the distant airways further demonstrated that, in chicks treated with the neuraminidase inhibitor, the air capillaries were distended and...
that the elastic fiber network, normally encasing these structures, was fragmented and diffuse (Fig. 5D). Comparison of transversal sections of thoracic aortas also showed the inhibitory effect of the sialidase inhibitor on vascular elastogenesis. In contrast to aortas from solvent-treated chicken embryos demonstrating compact concentric elastic membranes (Fig. 5E), aortas from sialidase inhibitor-treated embryos demonstrated a marked separation of elastic lamellae throughout the entire tunica media (Fig. 5F). The inhibitory effect of the sialidase inhibitor on elastogenesis (most effective at concentrations of 250 μM) initially established in cultures of human ASMCs was fully confirmed in cultures of other elastoblasts, such as normal skin fibroblasts and elastic cartilage chondroblasts (Fig. 6C).

**Treatment with Exogenous Sialidase Enhances Elastogenesis**—We have also established that treatment of all these cell types with exogenous sialidase from *C. perfringens*, which shares substrate specificity and extensive sequence homology with human lysosomal Neu1, induced a significant increase in the net deposition of insoluble elastin (Fig. 6A). The most pronounced elastogenic effect was observed when the exogenous enzyme (100 milliunits/ml) was added three times (on days 1, 3, and 5) during the course of 7-day cultures of all tested elastoblasts. Sialidase at <100 milliunits/ml produced only moderate elevations of insoluble elastin levels compared with untreated cultures, and exogenous sialidase at >100 milliunits/ml did not induce any further increase in a net elastin deposition. Northern blot analysis of parallel cultures showed that treatment of all tested cell types with exogenous sialidase or the sialidase inhibitor ddNeuAc did not modulate the level of message encoding tropoelastin (Fig. 6B).

The opposite effects of the endogenous sialidase inhibitor and exogenous sialidase on the net production of immunodetectable elastic fibers were further confirmed by immunofluorescence microscopy (Fig. 6C).
Sialidase Facilitates Elastic Fiber Assembly

FIGURE 7. Representative electron micrographs depicting 7-day cultures of human ASMCs immunostained with polyclonal anti-elastin antibody (labeled with 10-nm gold particles). Although control ASMCs produced a well polymerized core of elastic fiber densely decorated with gold-labeled anti-tropoelastin antibody, cells treated with ddNeuAc (ddNANA) were surrounded only with the microfibrillar scaffold of elastic fiber scarcely decorated with immunolabeled tropoelastin. ASMCs maintained in the presence of C. perfringens sialidase produced fibers containing more elastin compared with untreated control cells.

FIGURE 8. The results of morphometric analysis of 7-day cultures of dermal fibroblasts indicate that treatment with exogenous sialidase or the sialidase inhibitor ddNeuAc (ddNANA) selectively modulated deposition of immunodetectable elastin, but not other tested ECM components (fibrillin-1, fibronectin, and collagen type 1).

6C) and by immunoelectron microscopy (Fig. 7). Treatment with ddNeuAc completely prevented assembly of extracellular elastic fibers, whereas addition of exogenous sialidase enhanced deposition of elastic fibers in all tested cultures. The results of morphometric analysis of 7-day cultures of dermal fibroblasts indicated that treatment with exogenous sialidase or the sialidase inhibitor ddNeuAc selectively modulated deposition of immunodetectable elastin, but did not affect deposition of other ECM components: fibrillin, collagen type I, and fibronectin (Fig. 8).

Treatment with Exogenous Sialidase and Transfection with Neu1-expressing Plasmid Reverse Impaired Elastogenesis in Fibroblasts Derived from Patients with Sialidosis and Galactosialidosis—Additional results supporting the involvement of sialidase activity in elastic fiber assembly came from the experimental model involving human skin fibroblasts derived from patients with congenital diseases caused by primary (sialidosis) and secondary (galactosialidosis) deficiencies of Neu1. We demonstrated that 7-day cultures of dermal fibroblasts derived from sialidosis and galactosialidosis patients deposited significantly less metabolically labeled insoluble elastin compared with normal fibroblasts. At the same time, cultures of sialidosis and galactosialidosis fibroblasts demonstrated higher than normal levels of soluble (non-assembled) tropoelastin. Notably, the impaired elastogenesis observed in cultures of sialidosis and galactosialidosis fibroblasts could be reversed after addition of exogenous sialidase to the culture medium (Fig. 9A). Northern blot analysis of parallel 7-day cultures demonstrated that exogenous sialidase did not affect the steady-state levels of mRNA encoding tropoelastin (Fig. 9B). In all experiments, the sialidase-dependent increase in the levels of insoluble elastin was associated with a consequent decrease in the levels of immunoprecipitable tropoelastin. Exogenous sialidase did not, however, improve the impaired deposition of insoluble elastin in cultures of fibroblasts derived from patients with GM1 gangliosidosis, bearing a nonsense mutation of the β-galactosidase gene and consequently deficient in S-gal/EBP, which normally delivers tropoelastin to the cell surface (Fig. 9A).

Another strong endorsement supporting a role of Neu1 in elastic fiber assembly came from a set of experiments demonstrating that transduction of sialidosis fibroblasts with a Neu1/PPCA-expressing cDNA plasmid significantly improved deposition of metabolically labeled insoluble elastin (Fig. 10A) and immunodetected elastic fibers (Fig. 10, B and C). Interestingly, additional treatment of Neu1/PPCA-transduced fibroblasts with the sialidase inhibitor ddNeuAc nearly abolished the transduction-induced elastogenesis.

Sialic Acid Residues and Galactosugars Co-localize on a Microfibrillar Scaffold of Elastic Fibers—The final experiments were aimed at confirmation of our hypothesis that Neu1-dependent removal of sialic acid residues from glycoproteins forming the microfibrillar scaffold of elastic fibers would reveal the penultimate galactosugars capable of interaction with EBP. We tested 3-day cultures of human ASMCs (the stage at which the newly produced microfibrils are not fully embedded in the elastin core). Our results showed that both sialic acid residues (detected with rhodamine-labeled LFA lectin) and galactosugars (detected with fluorescein-labeled PNA lectin) co-localized on microfibrillar networks, detected with antibody to fibrillin-1 (Fig. 11, A and B). We then demonstrated that a 1-h exposure of 3-day confluent cultures of ASMCs to exogenous sialidase at 100 milliunits/ml of culture medium caused a complete removal of sialic acid residues from the microfibrillar scaffold of elastic fibers and revealed galactosugars (Fig. 11C).

DISCUSSION

The sialidases (neuraminidases) are widely distributed in nature and have been identified in numerous viral, bacterial, fungal, protozoan,
avian, and mammalian species (63, 64). In mammalian cells, four genetically distinct neuraminidases (sialidases) that differ in their tissue distribution, subcellular localization, and substrate specificity have been characterized. They have been localized to lysosomes (Neu1) (59, 60), to the cytosol (Neu2) (67–72), to the plasma membrane (Neu3, also known as ganglioside sialidase) (71, 73, 75, 76), and to mitochondria (Neu4) (44, 45); but only Neu1, expressed in all mammalian tissues and active mostly toward sialylated glycoproteins, has been detected in a multiprotein complex with \( H_{9252} \)-galactosidase and PPCA (77–79).

The fact that 67-kDa EBP has been positively identified as an enzymatically inactive spliced variant of \( H_{9252} \)-galactosidase (S-gal) (25, 26) allowed us to speculate that this protein, similar to the enzymatically active lysosomal variant, may also form a molecular complex with \( \beta \)-galactosidase and PPCA (77–79).

The results of this study indicate that proteins co-purified with 67-kDa S-gal/EBP from the lysates of human fibroblasts on elastin affinity columns were immunoreactive on Western blots with antibodies raised against human Neu1 and PPCA. The anti-Neu1 antibody reacted with a 61-kDa protein and with a doublet localized at the 46–44 kDa range. This suggests that the elastin-binding complex may contain both precursor and processed mature Neu1. Similarly, anti-PPCA antibody recognized a 55-kDa protein (with a mass similar to that of the cathepsin A precursor) and 32- and 20-kDa species, suggesting the presence of a mature form of this enzyme consisting of two peptide chains (27, 46). Anti-Neu1 and anti-PPCA antibodies showed a pattern of immunolocalization on the surfaces of elastin-producing cells identical to that of an antibody recognizing EBP.

Both lysosomal Neu1 and PPCA have been previously immunolocalized to the cell surface of normal fibroblasts (28, 46), activated lymphocytes (80, 81), and neutrophils (82), but such unusual localization of these lysosomal enzymes has been attributed to mistargeting or to alternative transport involving their subsequent exocytosis and endocytosis. Our data suggest that the association of Neu1 and PPCA with S-gal is not coincidental and indicate that these two proteins can form a molec-

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**FIGURE 9.** A, the results of the quantitative assay of metabolically labeled insoluble elastin demonstrate that 7-day cultures of fibroblasts derived from sialidosis and galactosialidosis patients deposited significantly less insoluble elastin compared with normal fibroblasts. They also demonstrated higher than normal levels of soluble (non-assembled) tropoelastin. Sialidosis and galactosialidosis fibroblasts, as well as normal fibroblasts, significantly improved their deposition of insoluble elastin when maintained in the presence of exogenous sialidase. This sialidase-induced increase in the levels of insoluble elastin was associated with a consequent decrease in the levels of immunoprecipitable tropoelastin. Exogenous sialidase did not improve the impaired deposition of insoluble elastin in cultures of fibroblasts derived from patient with GM1 gangliosidosis, bearing a nonsense mutation (\( nm \)) of the \( \beta \)-galactosidase (\( \beta \)-Gal) gene and deficient in S-gal/EBP. B, Northern blot analysis of parallel 7-day cultures demonstrated that exogenous sialidase did not affect the steady-state levels of mRNA encoding tropoelastin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ular complex with 67-kDa S-gal that is purposely targeted to the cell surface instead of the lysosomes (Figs. 1–3). To further clarify this point, we have to stress that no sialidase activity or Neu1 protein could be detected in the conditioned media from fibroblasts and ASMC cultures. This excluded a slim possibility that some Neu1 immunodetected on the cell surface could be secreted or released from damaged or dead cells.

The association of Neu1 and PPCA with S-gal/EBP, which acts as a tropoelastin chaperone and facilitates its secretion and assembly (16–18, 25, 26), raised questions of whether these proteins detected in the cell surface-targeted complex may display their enzymatic activities and thereby affect elastogenesis. The detection of significant sialidase activity in the affinity-purified elastin-binding complex isolated from the plasma membrane fraction of normal human fibroblasts (Table 1) and the fact that the inhibitors of Neu1 significantly inhibited deposition of insoluble elastin in cultures of three types of human elastoblasts (Figs. 4–8) strongly suggested that the enzymatic activity of cell surface-exposed Neu1 may be essential for normal elastogenesis. This assumption was additionally confirmed by studies showing that treatment of chicken embryos with sialidase inhibitors consistently impaired deposition of elastic fibers in developing lungs and arteries (Fig. 5).

Despite the fact that, at this moment, we cannot exclude the possibility that the competitive sialidase inhibitors used in our experiments could also penetrate to the cell interior, we postulate that they inhibit only cell surface-exposed Neu1. Because these inhibitors (mimicking the sialic acids-bearing moieties) can be internalized only via the endo-

**FIGURE 10.** The results of a representative experiment demonstrate that transduction of sialidosis fibroblasts with a cDNA construct encoding lysosomal Neu1 and PPCA significantly improved deposition of metabolically labeled insoluble elastin (A) and immunodetected elastic fibers (B). Additional treatment of Neu1/PPCA-transduced fibroblasts with the sialidase inhibitor ddNeuAc (ddNANA) nearly abolished the transduction-induced elastogenesis. The representative high power electron micrographs show 7-day cultures of sialidosis fibroblasts immunostained with polyclonal antibody to tropoelastin (labeled with 5-nm gold particles) (C). Although vector-transduced fibroblasts produced a microfibrillar scaffold of elastic fiber scarcely decorated with immunolabeled tropoelastin, cells transduced with Neu1/PPCA cDNA produced elastic fibers, the central core of which contained well assembled elastin.
Sialidase Facilitates Elastic Fiber Assembly

FIGURE 11. A, the representative micrographs demonstrate that Galβ1–3GalNac residues labeled with fluorescein-conjugated PNA (green) and sialic acid residues labeled with rhodamine-conjugated LFA (red) co-localized on the microfibrillar network produced in 3-day cultures of human ASMCS. B, both sialic acid residues labeled with rhodamine-conjugated LFA and Galβ1–3GalNac residues labeled with fluorescein-conjugated PNA co-localized with microfibrils detected with antibody to fibrillin-1. C, left panel, fluorescence microscopy demonstrated co-localization (yellow) of PNA-labeled Galβ1–3GalNac residues and LFA-labeled sialic acid residues on the microfibrillar scaffold in control cultures. Right panel, in cultures treated with exogenous sialidase, the microfibrillar scaffold was decorated with the remaining (green) Galβ1–3GalNac residues only. Magnification is ×100.

These results indicate that the enzymatic activity of sialidase positively modulates the final assembly of tropoelastin into elastic fibers only in cells with adequate expression of S-gal/EBP, which normally delivers tropoelastin to the cell surface. Because the levels of soluble (immuno-precipitable) tropoelastin in cultures of sialidosidosis and galactosialidosidosis fibroblasts were always higher than those detected in cultures of normal fibroblasts and because treatment with exogenous sialidase induced not only an increase in deposition of insoluble elastin, but also a significant decrease in soluble metabolically labeled tropoelastin, we assume that Neu1-dependent desialylation of certain molecules on the cell surface and/or extracellular glycoproteins may be needed for the final effective assembly of tropoelastin on the microfibrillar scaffold, which is a prerequisite for its further polymerization into insoluble elastin.

Definitive confirmation of the Neu1 involvement in elastogenesis came from experiments showing that Neu1-deficient fibroblasts derived from sialidosidosis patients did not assemble elastic fibers in culture and that such impaired elastogenesis could be reversed after treatment with exogenous bacterial sialidase or after transfection of cells with the Neu1 gene. In fibroblasts derived from sialidosidosis patients, elastogenesis was impaired, and such impaired elastogenesis could be reversed after treatment with exogenous bacterial sialidase or after transfection of cells with the Neu1 gene. We therefore postulate that the enzymatic activity of sialidase positively modulates the final assembly of tropoelastin into elastic fibers only in cells with adequate expression of S-gal/EBP, which normally delivers tropoelastin to the cell surface. Because the levels of soluble (immuno-precipitable) tropoelastin in cultures of sialidosidosis and galactosialidosidosis fibroblasts were always higher than those detected in cultures of normal fibroblasts and because treatment with exogenous sialidase induced not only an increase in deposition of insoluble elastin, but also a significant decrease in soluble metabolically labeled tropoelastin, we assume that Neu1-dependent desialylation of certain molecules on the cell surface and/or extracellular glycoproteins may be needed for the final effective assembly of tropoelastin on the microfibrillar scaffold, which is a prerequisite for its further polymerization into insoluble elastin.

The results of previous studies indicate that binding of galactosugar-bearing moieties to the lectin domain of S-gal/EBP causes conformational changes in this protein, leading to its dissociation from tropoelastin and other components of the cell surface-immobilized complex (14, 15, 19). We have therefore suggested that the release of tropoelastin from its chaperone occurs upon interaction of EBP with galactosugars present in the carbohydrate chains attached to microfibrillar glycoproteins (18–20, 22, 23). Subsequent coordinated anchoring of tropoelastin molecules to adjacent sites located on the N-terminal fragments of fibrillin-1 and fibrillin-2 (4, 7, 11) allows for the close alignment of their multiple lysine-rich domains, which can be consecutively cross-linked by lysyl oxidase.

The data presented in this work implicating Neu1 activity in the process of elastic fiber assembly turned our attention to the fact that most of the oligosaccharide chains of matrix glycoproteins contain the terminal nonreducing units of ketosidically linked sialic acids (N-acetylneuraminides), which may obscure the adjacent galactosugar residues. Therefore, we now propose that the enzymatic removal of the terminal sialic acids from the carbohydrate chains of matrix glycoproteins constitutes a critical step for the subsequent exposure of the penultimate galactose residues, indispensable for interaction with the lectin domain of EBP, and the subsequent release of tropoelastin from its chaperone (Fig. 12). Although the Neu1-dependent desialylation of oligosaccharide chains protruding from microfibrillar glycoproteins would initiate the direct assembly of the released tropoelastin molecules upon the microfibrillar bundles and formation of the elastin core, enzymatic desialylation of other adjacent glycoproteins (including fibronectin) may in turn induce dissociation of the subsequently secreted EBP-tropoelastin complexes and promote homo-aggregation of hydrophobic tropoelastin to assure thickening and dendritic growth of elastic fibers. At this point, we cannot completely exclude the possibility that Neu1-dependent desialylation of matrix glycoproteins may also lead to the exposure of chondroitin sulfate proteoglycans (versican, biglycan, and decorin), which have also been localized in close association with the microfibrillar scaffold of elastic fibers (83–86) and can also induce release of tropoelastin from its EBP chaperone (18–23). Because association with PPCA is required for the stability and activity of β-galactosidase and Neu1 within the lysosomes (27, 46–50), we speculate that PPCA present in the cell surface-targeted complex with S-gal and Neu1 may also be responsible for stabilization of the entire elastin-binding complex.

Both Neu1 and PPCA are sorted from the trans-Golgi network to the endosomal pathway. Like the majority of soluble lysosomal luminal proteins, PPCA is targeted by the mannose 6-phosphate receptor (87), whereas intracellular targeting of Neu1 is mediated by adaptor proteins through a tyrosine-containing motif in its C terminus (28). In the lysosome, the two proteins undergo activation. Activation of PPCA involves...
activity, and are then sorted to the vesicles destined to the plasma membrane. Thus, PPCA may also be “indirectly” instrumental in the process of elastic fiber assembly. Such an involvement seems to be suggested by the fact that fibroblasts isolated from three galactosialidosis patients (primary deficient in PPCA but also demonstrating a secondary deficiency of Neu1) deposited significantly less insoluble elastin compared with fibroblasts from sialidosis patients (Fig. 9A).

In conclusion, our data suggest that, in addition to their role in the intralysosomal catabolism of sialylated macromolecules, Neu1 and PPCA are targeted to the cell surface as part of the EBP complex and facilitate assembly of the elastic fibers (Fig. 12). Although more experiments may be needed to clarify the mechanism of trafficking and initial assembly of the cell surface-targeted elastin-binding complex, we believe that the our data significantly contribute to the bulk of knowledge on the complicated process of elastic fiber formation and may potentially lead to development of therapy in selected elastinopathies.

Compelling data indicate that, apart of its chaperoning function for intracellular tropoelastin (16, 18), the described molecular complex consisting of S-gal-Neu1-PPCA can also interact with diverse peptides maintaining GXXPG sequences, including a “principal” VGVAPG ligand derived from elastin, when exposed on the cell surface and function as a signaling receptor (19, 22, 23). These receptor-transduced signaling pathways include activation of G-proteins and sequential activation of numerous tyrosine kinases, including the Ras-Raf-MEK1/2-ERK1/2 (where MEK is mitogen-activated protein kinase/extracellular signal-regulated kinase and ERK is extracellular signal-regulated kinase) phosphorylation cascade, as well as phosphorylation of β-tubulin, microtubule-associated protein-1, α-actin, and tropomin-T (65, 74). It has been shown that these signaling pathways induce proliferation, differentiation, and motility of numerous cell types that do not produce elastin (macrophages, lymphocytes, epithelial cells, and certain cancer cells) (22, 23, 66), but can also be detected in non-confluent cultures of fibroblasts and arterial smooth muscle cells (57, 58, 88, 66). We propose that the pro-mitogenic and pro-migratory signals triggered via the surface-residing S-gal-Neu1-PPCA molecular complex in these elastoblasts could be effectively triggered only in a particular phase of their life cycle in which these cells do not produce tropoelastin. We speculate that initiation of elastin gene expression in confluent cultures creates the competitive situation in which the intracellular S-gal-Neu1-PPCA complex binds the newly synthesized tropoelastin and subsequently chaperones it to the cell surface. Because the final stage of this process (release of tropoelastin from its chaperone) also involves dissociation of S-gal from its cell surface-anchored partners (Neu1 and PPCA), the eventual triggering of intracellular signals is no longer possible.

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