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HUMAN NEURAMINIDASE IS A 60 kDa-PROCESSING PRODUCT OF PROSAPOSIN

Michel Potier, Sonia Lamontagne, Lorraine Michaud and Julie Tranchemontagne

Service de Génétique Médicale, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec, Canada H3T 1C5

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Human neuraminidase was purified from placenta as part of a large molecular weight complex with lysosomal ß-galactosidase and carboxypeptidase. Passage of this purified complex through a sialic acid-affinity column (fetuin-agarose) retained a minor 60 kDa protein which was eluted with 100 mM *N*-acetylneuraminic acid. This 60 kDa protein is recognized in Western blots of the purified complex by an anti-prosaposin antibody which at the same time was able to inhibit neuraminidase activity in the preparation. Furthermore, probing of cultured skin fibroblasts of patients affected with neuraminidase deficiency using the anti-prosaposin antibody revealed an abnormal 57 kDa protein. These results indicate that the 60 kDa protein is derived from prosaposin and has the characteristics of a neuraminidase.

Neuraminidase (sialidase, acylneuraminyl hydrolase, EC 3.2.1.18) is a glycosidase which hydrolyzes terminal sialic acid residues from oligosaccharides and glycolipids. Although this enzyme has been well studied as one of the two protein components of the influenza virus membrane (1), it also has an important function in the lysosomes of mammalian cells. In man, the deficiency of neuraminidase in tissues is associated to sialidosis, a recessively inherited lysosomal storage disorder characterized by progressive neurological degeneration, myoclonus, skeletal changes and macular cherry-red spots (reviewed in 2). However, isolation and characterization of mammalian neuraminidase has been difficult because of its extreme lability and membrane-bound character. Some neuraminidase activity co-purifies with ß-galactosidase and other proteins from human placental extracts as part of a large molecular weight complex (the NGC-complex) (3,4) but the identity of the protein responsible for neuraminidase activity has been elusive. On the basis of immunological evidence, both 60 (5) and 46 kDa proteins (6)

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have been proposed as candidates for neuraminidase but the 46 kDa protein was subsequently identified as a *N*-acetyl-α-galactosaminidase (7). Recent affinity labeling experiments with a photoreactive neuraminidase inhibitor support the idea that neuraminidase corresponds to a 60 kDa-protein in the NGC-complex (8).

Prosaposin is the 70 kDa precursor of four smaller 10 kDa activator proteins which functions are to increase the hydrolysis of sphingolipids by a variety of lysosomal hydrolases (9). In this paper, we consider prosaposin as a candidate for neuraminidase for the following reasons. First, the prosaposin gene (10) and that responsible for neuraminidase deficiency in man (11) were both localized on chromosome 10. Second, the primary structure of human (9) and rat prosaposin (also designated as the rat sulfated glycoprotein 1 of rat Sertoli cells) (12) showed significant similarity with the influenza virus neuraminidase (FLU NA) (13). Fourth, rat prosaposin was localized in the lysosomes (14). Fifth, we previously reported that prosaposin binds to a sialic acid-affinity column (fetuin-agarose) indicating that, like FLU NA, this protein has the capacity to recognize and bind to sialic acid residues (15). However, isolated prosaposin had no detectable neuraminidase activity (15). Thus, prosaposin may need proteolytic processing to express neuraminidase activity and/or interaction with other proteins. van der Horst et al. (5) reported that the interaction of neuraminidase with other components of the NGC-complex is necessary for enzymic activity. Our strategy to identify neuraminidase among the proteins of the NGC-complex was to directly probe active neuraminidase using a specific anti-prosaposin antibody. We report in this paper that a 60 kDa protein with neuraminidase activity is recognized by the antibody.

MATERIALS AND METHODS

Purification of the neuraminidase-β-galactosidase-carboxypeptidase (NGC) complex. The NGC-complex was purified from the human placenta according to a modification (4) of the method of Verheijen et al. (3). The glycoproteins of the placental extract were purified by chromatography on a concanavalin A-Sepharose (Sigma) column. To generate neuraminidase activity, the glycoprotein fraction was concentrated to about 40 mg/ml and incubated for 90 min at 37°C (3). The NGC-complex was then purified by affinity chromatography on a *p*-aminophenyl-β-D-thiogalactopyranoside-agarose (Sigma) column which specifically retained the β-galactosidase and associated proteins. The specific activities of neuraminidase, β-galactosidase and carboxypeptidase in the purified NGC-complex preparation are given in Table 1.

The purified NGC-complex was further fractionated on a fetuin-agarose (Sigma) column as previously described for prosaposin (15). The purified NGC-complex at 0.27 mg/ml was stirred gently overnight at 4°C with 2 ml of fetuin-agarose in 0.02 M sodium acetate buffer, pH 5.2, 0.1 M NaCl. The suspension was poured into a column and washed with the elution buffer (10 ml). The sialic acid-binding proteins were then eluted with 100 mM *N*-acetylneuraminic acid (NANA). The preparation was dialyzed against the acetate buffer and concentrated by ultrafiltration in an Amicon cell (PM-10 membrane).

Enzyme assays. The neuraminidase and ß-galactosidase activities were determined according to published procedures using the corresponding fluorogenic 4-methylumbelliferyl-glycoside derivatives as substrates (16,17). The carboxypeptidase activity was determined according to Tranchemontagne et al. (18). Proteins were assayed according to Bradford (19) with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Electrophoretic analysis of proteins was performed on SDS-polyacrylamide gel under reducing conditions according to Laemmli (20). The proteins were silver stained by the method of Merrill et al. (21).

After electrophoresis, the proteins were electrotransfered from the SDS-gel to a nitrocellulose filter (Schleicher and Schuell) for 3 h at 70 V, and immuno-detection was carried out according to Dewji et al. (22) using an anti-rat prosaposin immunoglobulin preparation (purified by Protein A-agarose affinity chromatography) diluted 1/500 in 0.1 M sodium phosphate buffer, pH 7.5, 0.15 M NaCl (PBS) containing 1% (w/v) bovine serum albumin (BSA). It was previously established that both the anti-human and anti-rat prosaposin immunoglobulins cross-reacted with human prosaposin on the immunoblots (15). Thus, the anti-rat prosaposin immunoglobulin preparation was routinely used for immuno-detection of human prosaposin. After washing with PBS containing 0.1% (v/v) Triton X-100, the antigen-antibody complex was detected using a ^{125}I-labelled goat anti-rabbit immunoglobulin. The radioactivity was detected by autoradiography using two intensifying screens.

Inhibition of placental neuraminidase activity with anti-prosaposin antibody. The purified NGC-complex (15µg) was incubated for 4 h at 4°C in PBS/BSA with increasing quantities of anti-prosaposin antibody (1/50 in PBS/BSA) in a total volume of 100 µl. After incubation, the preparation was shaken, and neuraminidase and ß-galactosidase activities were determined as described above.

Cultured skin fibroblasts. Skin fibroblasts of patients affected with sialidosis (cell lines GM01718, GM02837 and GM02921 obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, N. J.) and controls were cultured in minimum essential medium supplemented with 10% (v/v) heat inactivated fetal calf serum without antibiotics. At confluence, the cells were scraped, washed with 0.15 M NaCl and directly homogeneized in Laemmli's loading buffer. The proteins were electrophoresed and immunoblotted as described above.

RESULTS

The purified NGC-complex was passed through a sialic acid-affinity column (fetuin-agarose) in order to purify the

neuraminidase. Table 1 shows the distribution of the various enzymic activities associated to the NGC-complex after affinity chromatography on the fetuin-agarose column. Most of the ß-galactosidase and carboxypeptidase activities were recovered in the column wash and very little activities were found in the NANA-eluted fraction. In contrast, the neuraminidase activity was almost completely lost. This loss of neuraminidase activity was not unexpected considering the extreme lability of neuraminidase and the necessary interaction with other protein components of the NGC-complex for expression of enzyme activity (5). However, comparison of the protein composition of the purified NGC-complex preparation applied to the fetuin-agarose column (Fig. 1, lane 1) with the column wash (lane 2) by SDS-polyacrylamide gel electrophoresis revealed that a 60 kDa protein was specifically retained by the affinity column. The fraction eluted with 100 mM NANA was enriched with the 60 kDa-protein (Fig. 1, lane 3). These results suggest that the 60 kDa-protein has affinity for the sialic acid residues of the fetuin-agarose column although the protein had no demonstrable neuraminidase activity (Table 1). The usual protein components of the NGC-complex were also found in the various fractions including a 76 kDa doublet which represents the immunoglobulin IgM (5), a large 66 kDa band identified as ß-galactosidase (3) and the 54 kDa protein as the precursor of the 32 and 20 kDa protomeric components of the carboxypeptidase (23). The minor band around 44 kDa was previously shown to represent N-acetyl-α-galactosaminidase (7).

Table 1. Affinity chromatography of the purified placental multi-enzyme complex containing neuraminidase, ß-galactosidase and carboxypeptidase on a fetuin-agarose column

Fraction	Proteins (mg)	Enzyme activity Total (nmoles/min)			Specific (nmoles/min/mg protein)		
		Gal	Neu	Carb	Gal	Neu	Carb
Purified complex	1.15	3,428	46	5,778	2,981	40.2	5,025
Column wash	0.86	2,637	2.0	2,594	7,625	6.6	14,556
NANA-eluted	0.03	ND	ND	66	ND	ND	2,192

Gal, ß-galactosidase; Neu, neuraminidase; Carb, carboxypeptidase; NANA, N-acetylneuraminic acid; ND, not detected.

The 60 kDa protein isolated by affinity chromatography is immunologically related to prosaposin because it was recognized on immunoblots by the specific anti-prosaposin antibody (Fig. 2). To directly test that the 60 kDa immunoreactive protein is related to neuraminidase, we used the anti-prosaposin antibody to demonstrate selective inhibition of neuraminidase in the purified preparation of placental NGC-complex (Fig. 3). It must be pointed out that it is direct neuraminidase inhibition due to antibody recognition and not precipitation that we are testing under our experimental conditions. Precipitation tests may be misleading since an antibody raised against a component of the NGC-complex may cause precipitation of associated neuraminidase activity together with the antigenic component. To ensure that neuraminidase inhibition is not artefactual, several control experiments were run. First, the ß-galactosidase was used as an internal control and was not inhibited by the antibody (Fig. 3). Second,

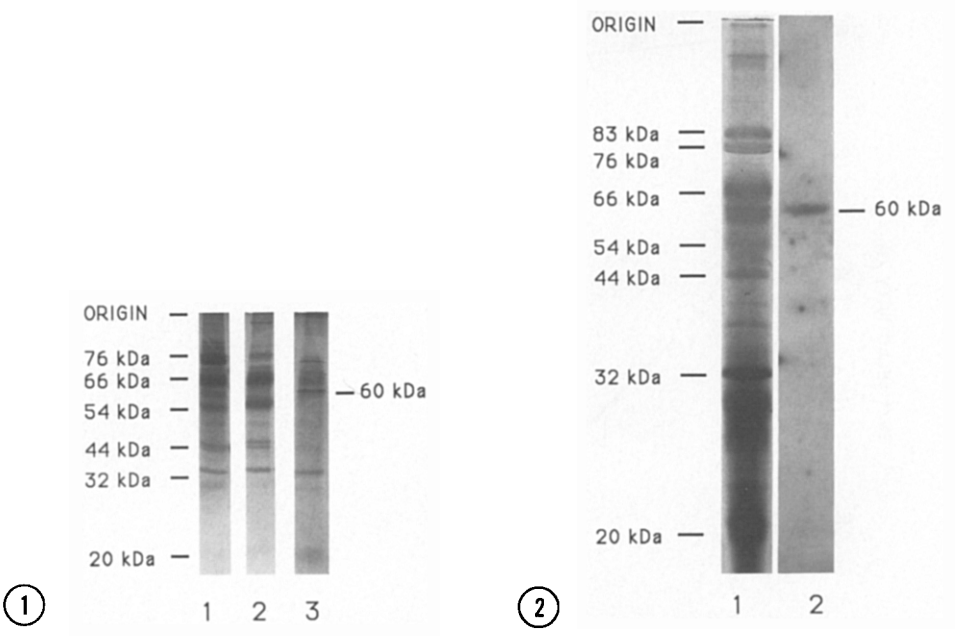
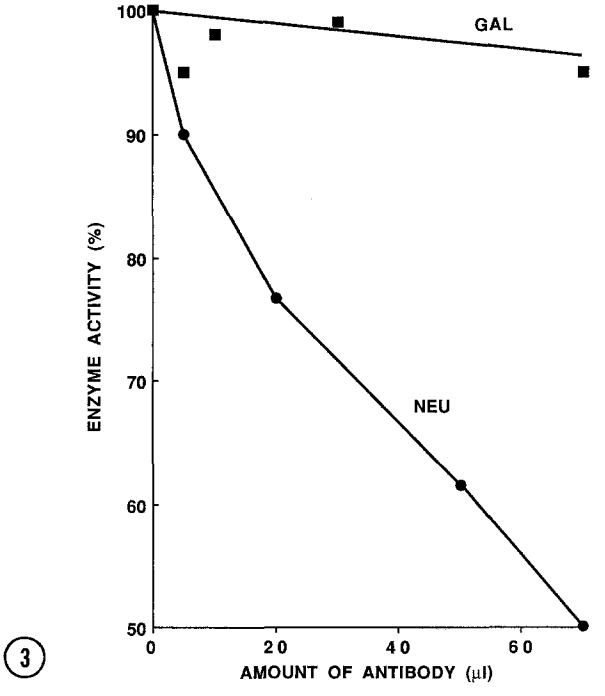


Fig. 1. SDS-polyacrylamide gel (12.5%, w/v) electrophoretic analysis of fractions obtained by chromatography of the purified placental complex on a fetuin-agarose affinity column. Lane 1, purified neuraminidase-ß-galactosidase-carboxypeptidase complex, 10 µg; lane 2, column wash, 10 µg; and lane 3, protein fraction eluted with 100 mM *N*-acetylneuraminic acid, 5 µg.

Fig. 2. SDS-polyacrylamide gel (11%, w/v) electrophoresis of purified placental neuraminidase-ß-galactosidase-carboxypeptidase multi-enzymic complex. Lane 1, silver stain of proteins; lane 2, immunoblot with anti-prosaposin antibody.

heat-denatured antibody (3 min at 100°C) had no inhibitory effect on neuraminidase indicating that the inhibition is not caused by heat-stable components in the immunoglobulin preparation. Third, other immunoglobulin preparations against unrelated proteins had no effect on neuraminidase activity of the NGC-complex (data not shown). Thus, we conclude that the anti-prosaposin antibody recognizes specifically the neuraminidase in the purified NGC-complex.

Final demonstration that the anti-prosaposin antibody recognizes neuraminidase was obtained by probing cultured skin fibroblast homogenates of patients affected with sialidosis (Fig. 4). In two patients among the three studied, we detected an abnormal 57 kDa protein band in addition to the normal 60 and 48 kDa bands detected in seven control cell lines. Variable levels of the 48 kDa band were found from one cell line to the other suggesting that it may represent a degradation product of



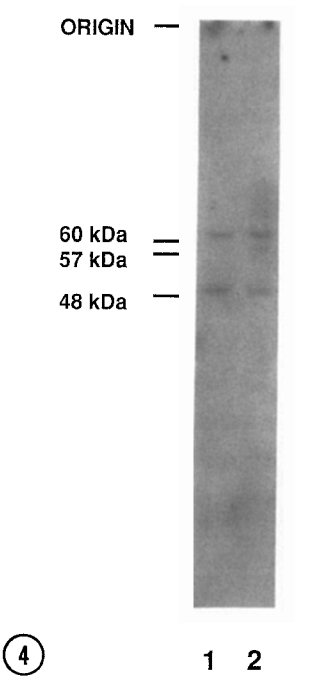


Fig. 3. Effect of increasing quantities of anti-prosaposin antibody on purified placental neuraminidase-ß-galactosidase-carboxypeptidase complex. Neuraminidase (NEU) and ß-galactosidase (GAL) activities.

Fig. 4. Immunoblots of cultured skin fibroblasts of a normal control (lane 1) and a patient affected with sialidosis (lane 2) with anti-prosaposin antibody. 10 µg of protein were deposited in each lane.

the 60 kDa band. These results suggest that an allelic mutation common to these two patients caused the synthesis of an abnormal smaller protein or produced an abnormal degradation product of neuraminidase.

DISCUSSION

We identified in the purified NGC-complex of the human placenta a minor 60 kDa-protein which binds to a sialic acid-affinity column (Fig. 1), and is immunologically related to prosaposin and neuraminidase. The anti-prosaposin immunoglobulin recognized the 60 kDa protein on immunoblots of the purified NGC-complex (Fig. 2) and at the same time specifically inhibited the neuraminidase activity (Fig. 3). These findings agree with previous immunological studies of van der Horst et al. (5) who identified neuraminidase as a 60 kDa protein. In addition, photo-affinity labelling of the NGC-complex with a potent neuraminidase inhibitor specifically labelled a protein in the 60 kDa range (8). Estimates of the molecular size of human placental neuraminidase by the radiation inactivation method also gave values in the 60 kDa range, between 63 and 67 kDa (24). Finally, the identification of an abnormal immunoreactive protein in the cultured fibroblasts of patients affected with sialidosis (Fig. 4) indicates that the 60 kDa-protein characterized in this paper is the neuraminidase.

The human neuraminidase may have a similar folding structure to that of FLU NA made of six-fold repeated ß-sheets (each ß-sheet composed of four short strands) placed in a fan-like arrangement around a pseudo-six-fold axis of symmetry (1). We have proposed a similar structure for prosaposin but with eight repeated ß-sheets instead of six (15). In our model, the amino acid sequence of the first six putative *N*-terminal ß-sheets of prosaposin are aligned with the six ß-sheets of FLU NA. Therefore, it seems that the activation process of prosaposin into a neuraminidase involves the proteolytic removal of the two C-terminal ß-sheets of the prosaposin precursor.

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