Cellular localization of neuraminidases in cultured human fibroblasts

Marcia ZEIGLER and Gideon BACH*

Department of Human Genetics, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

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The cellular localization of glycoprotein and ganglioside sialidases in normal and I-cell-disease cultured fibroblasts has been investigated. Cellular organelles have been separated on a colloidal silica gradient. The subcellular distribution of these enzymes indicated that the glycoprotein sialidase is mainly a lysosomal hydrolase, whereas the ganglioside sialidase is primarily located in the plasma membranes. The latter isoenzyme is tightly bound to these membranes and thus could not be extracted by homogenization in the presence of Triton X-100. The interpretation of this finding and its relation to the pathochemistry of sialidase-deficient disorders is discussed.

Mammalian neuraminidases (sialidase, α -N-acetylneuraminy] glycohydrolase, EC 3.2.1.18) have been studied primarily in brain tissues (Carubelli *et al.*, 1962; Leibovitz & Gatt, 1968; Roukema & Heijlman, 1970; Venerando *et al.*, 1975; Sandhoff & Pallmann, 1978), and to a lesser extent in other tissues such as kidneys (Van Dijk *et al.*, 1976), liver (Kean & Bighouse, 1974) and cultured fibroblasts (Schengrund *et al.*, 1973; Caimi *et al.*, 1979; Thomas *et al.*, 1979; Lowden & O'Brien, 1979). These enzymes have been found in neurons and localized primarily to plasma membranes and smooth microsomes (Schengrund & Rosenberg, 1970; Tettamanti *et al.*, 1972, 1973). They presumably function physiologically in the cellular transport of macromolecules and the maturation of glycoproteins. Although these enzymes have never been sufficiently purified, it has become increasingly apparent that the enzyme(s) active towards glycolipids (gangliosides) and those hydrolysing sialic acid residues from glycoproteins are two different enzymes (Caimi *et al.*, 1979). This is further emphasized by the fact that the glycoprotein sialidase is specifically deficient in various forms of sialidosis (Cantz *et al.*, 1977; Kelly & Graetz, 1977; Lowden & O'Brien, 1979), whereas the ganglioside sialidase in these disorders is normal (Lowden & O'Brien, 1979; Cantz & Messer, 1979). On the other hand, in mucopolipidosis type IV, the ganglioside sialidase is the isoenzyme specifically affected (Bach *et al.*, 1979). Synthetic substrates such as neuraminy]lactose, methoxyphenylneuraminic acid and 4-methylumbelliferyl-sialic acid have proved useful in the assay of glycoprotein sialidase (Tettamanti *et al.*, 1973; Lowden & O'Brien, 1979; Cantz & Messer, 1979). The ganglioside sialidase can at present only

be assayed using its natural gangliosides as substrates.

Findings of Strecker *et al.* (1977) have suggested the existence of more than one glycoprotein sialidase, each specific for a different glycosidic bond, but this has never been adequately confirmed. Although there is no proof for the existence of more than one ganglioside sialidase in mammalian tissues, this enzyme has been localized to various subcellular organelles. Subcellular fractionation of neurons has shown that the majority of ganglioside sialidase is found in the plasma membrane (nerve endings) and microsomal fractions, with only minor activities found in the lysosomes and cytosol (Carubelli *et al.*, 1962; Leibovitz & Gatt, 1968; Roukema & Heijlman, 1970; Tallman *et al.*, 1972; Tettamanti *et al.*, 1973; Venerando *et al.*, 1975). The distribution of this enzyme has not been sufficiently characterized in extraneuronal cells.

In the present investigation we report the subcellular distribution of sialidases in normal and I-cell disease (mucopolipidosis II) cultured fibroblasts. I-cell disease is characterized by the intracellular deficiency of most lysosomal hydrolases (Leroy *et al.*, 1971). Previous findings have demonstrated a total deficiency of glycoprotein sialidase in I-cell disease, whereas ganglioside sialidase was only slightly affected (Bach *et al.*, 1979). The deficiency of glycoprotein sialidase has been suggested to be involved in the defect of this disease (Thomas *et al.*, 1976; Strecker *et al.*, 1976).

Materials and methods

Cell culture

Cultured skin fibroblasts, obtained from healthy controls and an I-cell disease patient were propagated in 75 cm² or 150 cm² plastic tissue culture

* To whom reprint requests should be addressed.

flasks (Falcon) to a cell density of $(5-7) \times 10^6$ cells/75 cm² flask. The culture conditions have been described previously (Bach *et al.*, 1977). Cells were harvested 10–14 days after the last passage.

Enzyme assay

Ganglioside sialidase was determined using pure ganglioside G_{M3} or mixed bovine brain gangliosides (Sigma; type II) as substrates. Neuraminyl-lactose (Sigma, type II) served as a substrate for glycoprotein sialidase. Details of the sialidase determinations have been described previously (Bach *et al.*, 1979). 5'-Nucleotidase (EC 3.1.3.5; plasma-membrane marker) and α -mannosidase (EC 3.2.1.24; lysosomal marker) were determined as previously described (Bach & Liebmann, 1979). Arylsulphatase C (EC 3.1.6.1), used as the microsomal marker, was determined using 4-methyl-umbelliferyl sulphate (Koch-Light) as substrate (Meyer *et al.*, 1979). Galactosyltransferase (EC 2.4.1.38; Golgi-apparatus marker) was determined by the method of Rome *et al.* (1979).

Subcellular fractionation

Subcellular fractionation was performed on a colloidal silica gradient as described by Rome *et al.* (1979). The starting density of the colloidal silica was 1.065–1.069 g/ml with an osmolarity of 330 m-osm and a conductivity of 1.00 m-mho. Colloidal silica (30 ml) was layered on a 4 ml cushion of super-saturated sucrose (density 1.2 g/ml) in a 40 ml quick-seal tube (Beckman). Post-nuclear supernatant (3–4 ml) containing 7 mg of protein in 0.25 M-sucrose was layered on the colloidal silica. The gradient was generated in a Beckman ultracentrifuge (model no. L5-50B) at a speed of 20000 rev./min for 2.5 h using a vertical rotor (VTi-50). Fractions (0.5 ml) of the generated gradient were collected from the bottom of the centrifuge tube. By this gradient, lysosomes are distributed in two peaks, one found at a density of 1.085 g/ml and the second at 1.11. Plasma-membrane fragments are found at a density of 1.080 and microsomes and Golgi at 1.085 (Rome *et al.*, 1979). In some experiments, the dense lysosomal and the

Fig. 1. Distribution pattern of glycoprotein sialidase (a) and ganglioside sialidase (b) of post-nuclear supernatant on colloidal silica gradient

The distribution of the different markers in (a) is identical with that shown in (b). The densities of the peak activities of the different markers are identical with those reported by Rome *et al.* (1979) (see the Materials and methods section). O·····O, α -Mannosidase; x, galactosyltransferase; O-----O, arylsulphatase C; □, 5'-nucleotidase; ●, ganglioside sialidase; △, neuraminyl-lactose sialidase.

buoyant (Golgi, microsomes, 'light' lysosomes and plasma-membrane fragments) fractions were pooled separately, diluted (1:1) with physiological saline and concentrated by centrifugation at 100000g for 1.5 h. The particles that were layered above the silica were collected, packed by centrifugation for 0.5 h, at 100000g and resuspended in 0.5 ml of saline. Attempts to solubilize the enzymes from the particles in the buoyant and the dense lysosomal fractions were carried out by ultrasonication (330S Braunsonic sonicator; small probe B; Braun) in the presence of 0.1% Triton X-100.

Results

The distribution of ganglioside and neuraminyl-lactose sialidases in normal fibroblasts throughout the generated gradient is presented in Fig. 1. Neuraminyl-lactose sialidase is primarily a lysosomal enzyme since its activity runs parallel to the lysosomal marker α -mannosidase. Some activity may be associated with the plasma membranes, although this is impossible to assess quantitatively due to the overlap of the different particles in the buoyant fraction. Most of the ganglioside sialidase, on the other hand, is associated mainly with the plasma membranes. Since the differences in densities of microsomes and plasma membranes are too small for their adequate separation by the present gradient, the presence of some ganglioside sialidase in the microsomes cannot be excluded. Only trace activities of ganglioside sialidase were found in the

dense lysosomal fraction. Pooling of the dense lysosomal fraction with subsequent concentration (see the Materials and methods section) indicated that about 5% of the total ganglioside sialidase activity is found in this fraction. Concentration of the buoyant fraction, with subsequent sonication in the presence of 0.1% Triton X-100, did not release significant amounts of ganglioside sialidase into the supernatant (Table 1). By these conditions, most of the arylsulphatase C and α -mannosidase could be solubilized, whereas only 20% of the 5'-nucleotidase was found in the supernatant. This further reinforces the localization of most of the ganglioside sialidase to the plasma membranes.

I-cell-disease fibroblasts showed a similar ganglioside sialidase distribution to normal cells (Fig. 2), although the peak activity is slightly decreased. The enzyme deficiencies in I-cell disease are thought to be due to a failure of the lysosomal enzymes to undergo packaging to mature lysosomes by the post-synthetic apparatus (Sly & Stahl, 1978). The present finding of diminished activity of α -mannosidase in the dense lysosomal fraction (mature lysosomes) and the presence of α -mannosidase in the buoyant fraction, which contains the newly synthesized enzyme (Rome *et al.*, 1979), is in agreement with this.

Discussion

The important functions that sialidases have in the normal metabolism of glycoproteins and glycolipids and their involvement in certain inborn errors of metabolism emphasizes the need for more detailed study and characterization of these enzymes in human cells. Up until now these enzymes have not been characterized sufficiently in extraneuronal tissues so that the aetiology of sialidase-deficient diseases is not always clear. In the present study the glycoprotein sialidase has been localized primarily to the lysosomal fraction in cultured skin fibroblasts. Therefore, the deficiency of this enzyme in I-cell disease (Bach *et al.*, 1979) correlates well with the

Table 1. Solubilization by Triton X-100 of enzymes from the buoyant particles

Enzyme	Solubility (%)
α -Mannosidase	70
Arylsulphatase C	66
5'-Nucleotidase	20
Ganglioside sialidase	10

Fig. 2. Distribution of ganglioside sialidase in cultured fibroblasts of I-cell disease patient. The distribution of the various markers and the units of enzyme activities are as described in Fig. 1. ○, α -Mannosidase; ●, ganglioside sialidase.

deficiency of most other lysosomal hydrolases in this disease. The ganglioside sialidase, on the other hand, was shown to be localized mainly in the plasma membranes with only trace activities found in lysosomes. In the light of these and previous (Bach *et al.*, 1979) data, the presence of ganglioside sialidase in I-cell disease fibroblasts is to be expected.

In the various forms of sialidosis, the glycoprotein sialidase is the only enzyme specifically affected, leading to the intralysosomal accumulation of sialic acid-rich glycoproteins. Allelism in the glycoprotein sialidase locus is the most plausible explanation for the clinical heterogeneity of this group.

The presence of ganglioside sialidase in the external surface of plasma membranes in cultured fibroblasts has been suggested in previous reports (Schengrund *et al.*, 1973; Yogeewaran & Hakomori, 1975). These studies have shown that the enzyme disappears once the cultures have reached confluency. This phenomenon has been attributed to cell-contact inhibition (Yogeewaran & Hakomori, 1975). Whether this enzyme is inactivated at this stage of cellular growth or merely redispersed in the membrane, so it could not be detected by the techniques used, has never been answered. Our findings have localized this enzyme to plasma membranes in confluent cell cultures, indicating that this enzyme is present in the plasma membranes also at confluency. Further studies, regarding the transposition of this enzyme in plasma membranes of cultured fibroblasts during the various stages of cellular growth, would certainly be enlightening.

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