CMP-sialic acid, the sole sialosyl donor, is intra-axonally transported

Michihiro Igarashi, Yoshiaki Komiya and Masanori Kurokawa

Department of Biochemistry, Institute of Brain Research, Tokyo University Faculty of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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N-Acetyl-D-[6-^{3}H]mannosamine was injected into the 9th dorsal root ganglion of *Xenopus laevis* and the intra-axonal transport of chloroform/methanol-soluble radioactivity was analyzed using thin-layer chromatography coupled with fluorography. Three radioactive groups were distinct in consecutive segments of the sciatic nerve. The first is due to *N*-acetyl-D-mannosamine itself which labels the nerve uniformly, but does not seem to migrate within axons. The second group, representing most probably CMP-sialic acid, migrates at about 8 mm/day at 15°C. The third is a species of ganglioside uniquely present in the frog nerve, and this migrates at 1–3 mm/day. Our demonstration of the intra-axonal transport of CMP-sialic acid affords direct support to the contention that sialosylation of the ganglioside can occur in axon terminals.

Axonal transport *CMP-sialic acid* *Ganglioside* N-*Acetylmannosamine*

1. INTRODUCTION

Recent studies have shown that gangliosides may play a significant role in the regulation of cell-surface events [1]. Gangliosides occur at high concentrations in the neuronal membrane, suggesting their importance in neuronal function [2,3]. Several lines of evidence indicate that gangliosides are transported within axons after their synthesis in neuron soma [4–7]. On the other hand, there are some indications that a portion of gangliosides is synthesized locally at the external cell surface and/or in nerve endings [8–11]. In this study, both CMP-sialic acid and a species of ganglioside were found to migrate intra-axonally at rates distinct from each other. The results suggest that sialosylation of gangliosides occurs not only in Golgi regions but also in axon terminals.

Abbreviations: ManNAc, *N*-acetyl-D-mannosamine; NeuAc, *N*-acetylneuraminic acid; TLC, thin-layer chromatography; C-M, chloroform-methanol

2. MATERIALS AND METHODS

2.1. *Chemicals*

The following chemicals were obtained from the sources indicated: [6-^{3}H]ManNAc (26.6Ci/mmol), New England Nuclear (Boston, USA); CMP-[4,5,6,7,8,9-^{14}C]NeuAc (35mCi/mmol), Amersham (Bucks, England); non-labeled CMP-NeuAc, Sigma (St. Louis, USA); neuraminidase from *Vibrio cholerae*, Calbiochem (La Jolla, USA); high-performance TLC plates with concentration zone (silica gel 60, 10 × 20 cm), Merck (Darmstadt, FRG); Unisil (200–325 mesh), Clarkson Chemical (Williamsport, USA).

2.2. *Treatment of animals*

Adult female *Xenopus laevis*, weighing about 100 g, were anesthetized by intraperitoneal injection of 1 ml of 20% urethane. [^{3}H]ManNAc (80 μCi/0.4 μl frog saline) was injected into the 9th dorsal root ganglion as described [12]. After suture, the animals were kept in water at 15°C. At prescribed time intervals, a frog was decapitated

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and the sciatic nerve, together with the DRG, was removed and cut into consecutive 5 mm segments.

2.3. *Extraction, TLC and fluorography*

The ganglion and each nerve segment were extracted with 0.6 ml C-M (2:1, v/v) and then with an equal volume of C-M (1:2, v/v). After washing the residue with methanol, the combined extract was centrifuged at 18000 × *g* for 20 min. The supernatant was evaporated under a stream of nitrogen gas, and the dried sample dissolved in a small volume of C-M (1:1, v/v), applied to the TLC plate and developed by C-M/5 N NH_4OH/0.4% $CaCl_2$ (60:40:4:5, v/v; solvent A) [13]. After drying, the plate was sprayed with 0.4% 2,5-diphenyloxazole in 2-methylnaphthalene (containing 10% toluene) [14], and exposed to Kodak XAR-5 X-ray film at −70°C.

2.4. *Treatment with acid or neuraminidase*

One half of the C-M extract from one segment was evaporated and incubated in 0.2 ml H_2SO_4 at 37°C for 60 min. The other was treated with neuraminidase according to Iwamori and Nagai [15]. The treated sample was lyophilized, applied to the TLC plate and developed with ethanol-1 M CH_3COONH_4 (3:2, v/v; solvent B) [16]. The plate was then subjected to fluorography as above.

2.5. *Isolation of gangliosides from frog sciatic nerve*

Sciatic nerves from 20 animals were frozen by liquid nitrogen, crushed to powder, and extracted with 20 vols (wet wt) of C-M (2:1, v/v), and then with an equal volume of C-M (1:2, v/v). To isolate the gangliosides, the crude extract was subjected to DEAE-Sephadex column chromatography, Sep-Pak C_{18} cartridge, and Unisil column chromatography as described by Ledeen and Yu [17].

3. RESULTS AND DISCUSSION

Three radioactive bands were seen in the sciatic nerve 5 days (fig.1a) and 8 days (fig.1b) after injecting [^{3}H]ManNAc into the ganglion. Band A was detectable by fluorography even 3–18 h after the injection (not shown). In TLC, band A comigrated with authentic [^{3}H]ManNAc. Ligation of the nerve performed shortly after isotope injection did not cause any accumulation of the [^{3}H]ManNAc radioactivity on either side of the ligature. Band A was thus concluded to represent [^{3}H]ManNAc per se, which uniformly labeled a long stretch of the nerve most probably via general circulation.

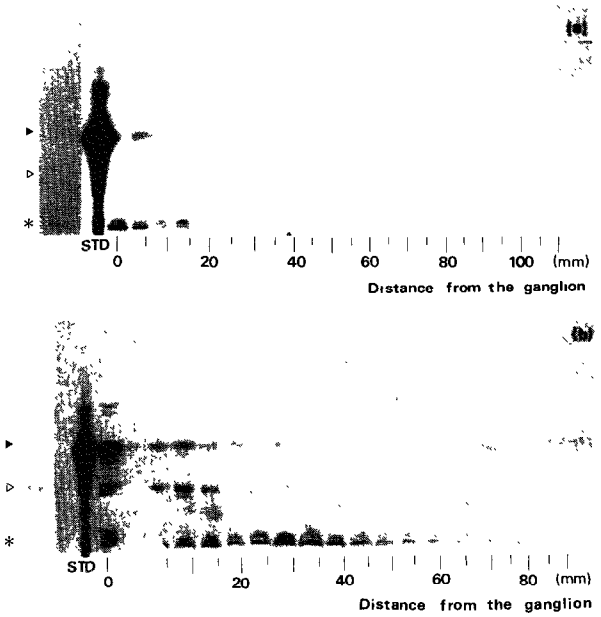

Fig.1. Distribution of radioactivity in frog sciatic nerve 5 days (a) and 8 days (b) after injecting [^{3}H]ManNAc into the 9th dorsal root ganglion of *X. laevis*. Extracts of consecutive 5 mm nerve segments were subjected to TLC in solvent A and the radioactivity was subsequently visualized by fluorography. Bands A (►), B (▷) and C (*) show mobilities identical with [^{3}H]ManNAc, a ganglioside purified from frog nerve, and CMP-sialic acid, respectively. The position of CMP-sialic acid visualized by resorcinol-HCl reagent is shown on the left in (a), and that of a purified nerve ganglioside on the left in (b). STD, [^{3}H]ManNAc.

Band B was observed in the first segment on day 5 (fig.1a) and in the first 3 segments on day 8 (fig.1b), indicating a migration rate of 1–3 mm/day. In TLC, band B comigrated with the major ganglioside in the *Xenopus* sciatic nerve (fig.1b). This ganglioside is considered to have a structure similar to that described in *Rana catesbeiana* [18].

The migrating front of band C, the most hydrophilic band amongst the three, reached the 8th segment on day 5 (fig.1a) and the 14th segment on day 8 (fig.1b). The rate of migration was thus about 8 mm/day.

Any band corresponding to band C was not detected when the TLC plate was stained with resorcinol prior to fluorography, indicating that the amount of band C in each nerve segment was too small to be detected by this conventional technique. Therefore, we had to use the following means to identify the chemical nature of this band.

The radioactivity associated with band C was predominantly recovered in the 5% trichloroacetic acid-soluble fraction (not shown). With solvent B, the R_f value for band C was 0.67, in contrast to 0.87 for band B, the major ganglioside in the sciatic nerve. R_f values for band C were identical to those of authentic CMP-sialic acid in TLC developed by 2 different solvent systems (figs 1a, 2 and 3). Treatment of band C with a weak acid caused a marked reduction in radioactivity comigrating with authentic CMP-NeuAc, and the appearance of radioactivity comigrating with authentic NeuAc (figs 2 and 3). In the case when band C was treated with neuraminidase from *V. cholerae*, the chromatographic mobility of the band C-associated radioactivity did not change (fig.2). CMP-sialic acid is known to be extremely acid-labile [19] and resistant to neuraminidase because of its β-sialosyl linkage [20]. All of these observations strongly suggest that band C represents CMP-sialic acid.

We have thus observed that a species of ganglioside, which is the main ganglioside in the frog sciatic nerve, migrates within axons after its sialosylation in the soma. Another striking finding in this study was the intra-axonal transport of CMP-sialic acid, the sole sialosyl donor synthesized exclusively in the cellnucleus [21–24]. The axonal transport of CMP-sialic acid lends direct support to the possibility that sialosylation of gangliosides (and also of sialo-glycoproteins) can occur in axon terminals [25], though a stringent demonstration of sialosyltransferases in synaptic plasma membranes awaits further study.

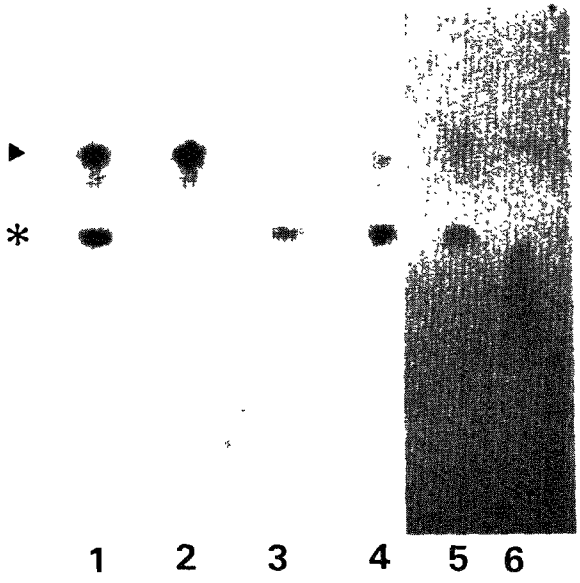

Fig.2. Treatment of band C (*) with a weak acid or neuraminidase from *V. cholerae*. Lanes: 1–4, fluorogram after TLC; 5–6, resorcinol stain after TLC. TLC plates were simultaneously developed with solvent B. Extract of 2nd nerve segment 4 days after injecting [^{3}H]ManNAc, before (lane 1) and after (lane 2) the acid treatment. Extract of 3rd segment from the same nerve, before (lane 3) and after (lane 4) the neuraminidase treatment. Lane 5, authentic CMP-NeuAc. Lane 6, authentic NeuAc.

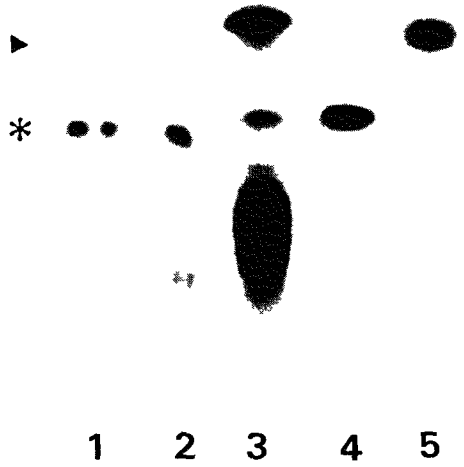

Fig.3. Acid hydrolysis of band C (*) and authentic CMP-[^{14}C]NeuAc. Extract of 5th nerve segment 14 days after injecting [^{3}H]ManNAc, before (lane 1) or after (lane 2) acid treatment. Lanes: 3, authentic CMP-[^{14}C]NeuAc after acid treatment; 4, CMP-[^{14}C]NeuAc; 5, [^{3}H]ManNAc. The TLC plate was developed with solvent B, and then fluorographed. The bands above CMP-[^{14}C]NeuAc in lanes 3 and 4 appear to be contaminants.

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REFERENCES

[1] Hakomori, S.-I. (1984) Trends Biochem. Sci. 9, 453–459.
[2] Ando, S. (1983) Neurochem. Int. 5, 507–537.
[3] Ledeen, R.W. (1984) J. Neurosci. Res. 12, 147–159.
[4] Forman, D.S. and Ledeen, R.W. (1972) Science 177, 630–633.
[5] Landa, C.A., Maccioni, H.J.F. and Caputto, R. (1979) J. Neurochem. 33, 825–838.
[6] Rösner, H. and Merz, G. (1982) Brain Res. 236, 63–75.
[7] Gammon, C.M., Goodrum, J.F., Toews, A.D., Okabe, A. and Morell, P. (1985) J. Neurochem. 44, 376–387.
[8] Matsui, Y., Lombard, D., Hoflack, B., Harth, S., Massarelli, R., Mandel, P. and Dreyfus, H. (1983) Biochem. Biophys. Res. Commun. 113, 446–453.
[9] Ledeen, R.W., Skrivanek, J.A., Tirri, L.J., Margolis, R.K. and Margolis, R.U. (1976) Adv. Exp. Med. Biol. 71, 83–103.
[10] Roseman, S. (1970) Chem. Phys. Lipids 5, 270–299.
[11] Preti, A., Forilli, A., Lombardo, A., Caimi, L. and Tettamanti, G. (1980) J. Neurochem. 35, 281–296.
[12] Abe, T., Haga, T. and Kurokawa, M. (1973) Biochem. J. 136, 731–740.
[13] Ariga, T., Sekine, M., Nakamura, K., Igarashi, M., Nagashima, M., Miyatake, T., Suzuki, A. and Yamakawa, T. (1983) J. Biochem. (Tokyo) 93, 889–893.
[14] Bonner, W.M. and Stedman, J.D. (1978) Anal. Biochem. 89, 247–256.
[15] Iwamori, M. and Nagai, Y. (1981) J. Biochem. (Tokyo) 89, 1253–1264.
[16] Carey, D.J. and Hirschberg, C.B. (1979) Biochemistry 98, 2086–2092.
[17] Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. 83, 139–191.
[18] Ohashi, M. (1982) Adv. Exp. Med. Biol. 152, 83–91.
[19] Comb, D.G., Shimizu, F. and Roseman, S. (1959) J. Am. Chem. Soc. 81, 5513–5514.
[20] Corfield, A.P. and Schauer, R. (1982) in: Sialic Acids. Chemistry, Metabolism and Function (Schauer, R. ed.) Cell Biology Monographs vol.10, pp.195–261, Springer, Wien.
[21] Kean, E.L. (1970) J. Biol. Chem. 245, 2301–2308.
[22] Van den Eijnden, D.H. (1973) J. Neurochem. 21, 949–958.
[23] Coates, S.W., Gurney, T. jr, Sommers, L.W., Yeh, M. and Hirschberg, C.B. (1980) J. Biol. Chem. 255, 9225–9229.
[24] Van Rinsum, J., Van Dijk, W., Hooghwinkel, G.J.M. and Ferwerda, W. (1981) Biochem. J. 210, 21–28.
[25] Tettamanti, G., Preti, A., Cestaro, B., Venerando, B., Lombardo, A., Ghidoni, R. and Sonnino, S. (1980) Adv. Exp. Med. Biol. 125, 263–280.