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Different reactivity to lysophosphatidylcholine, DIDS and trypsin of two brain sialyltransferases specific for *O*-glycans: a consequence of their topography in the endoplasmic membranes

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Some properties of two distinct rat brain sialyltransferases, acting on fetuin and asialofetuin, respectively, were investigated. These two membrane-bound enzymes were both strongly inhibited by charged phospholipids. Neutral phospholipids were without effect except lysophosphatidylcholine (lysoPC) which modulated these two enzymes in a different way. At 5 mM lysoPC, the fetuin sialyltransferase was solubilized and highly activated while the asialofetuin sialyltransferase was inhibited. Preincubation of brain microsomes with 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), known as a specific anion inhibitor and a non-penetrating probe, led to a moderate inhibition of the asialofetuin sialyltransferase just as in the case of the ovomucoid galactosyltransferase (used here as a marker for the luminal side of the Golgi membrane); under similar conditions, the fetuin sialyltransferase was strongly inhibited. In the presence of Triton X-100, which induced a disruption of membranes, all three enzymes were strongly inhibited by DIDS. Trypsin action on intact membranes showed that asialofetuin sialyltransferase, galactosyltransferase and fetuin sialyltransferase were all slightly inhibited. After membrane disruption by Triton X-100, the first two enzymes were completely inactivated by trypsin while the fetuin sialyltransferase was quite insensitive to trypsin treatment. From these data, we suggest that the fetuin sialyltransferase, accessible to DIDS, is an external enzyme, oriented closely towards the cytoplasmic side of the brain microsomal vesicles (endoplasmic and Golgi membranes), whereas the asialofetuin sialyltransferase is an internal enzyme, oriented in a similar manner to the galactosyltransferase. Morever, the anion site (nucleotide sugar binding site) of the fetuin sialyltransferase must be different from its active site, as this enzyme, when solubilized, is strongly inhibited by DIDS while no degradation is observed in the presence of trypsin.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; lysoPC, lysophosphatidylcholine; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Mes, 4-morpholineethanesulfonic acid.

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Introduction

The brain is known to possess high quantities of glycoconjugates: glycolipids such as gangliosides and cerebrosides, and soluble and membranous glycoproteins. It has been suggested that these molecules play a key role in cell-cell recognition and intracellular interactions [1]. These properties are of prime importance because of the abundance of intercellular contacts in this tissue. The biosynthesis of the oligosaccharide chains and study of cerebral glycosyltransferases and their regulation may contribute to the knowledge of the specific functions exerted in brain by glycoproteins. Glycoconjugates with NeuAc residues are located essentially in synaptic regions and have been postulated to be involved in the synaptic transmission [2]. A number of studies on sialyltransferases had previously been reported in rat brain by Van den Eijden and Van Dijk [3] and Ng and Dain [4] and in embryonic chicken brain by Den et al. [5]. However in brain, the determination of the specificity of one or more sialyltransferases was not always provided. In this paper, we have studied two sialyltransferases, each having a narrow specificity for one acceptor: one enzyme transfers NeuAc residues onto asialofetuin, the other onto fetuin. This last sialyltransferase, specific for *O*-glycans, has been demonstrated to be a NeuAcα(2-3)galactosylβ(1-3)*N*-acetylgalactosaminideα(2-6)-sialyltransferase [6], similar to the one described in fetal calf liver [7].

The main biological role of the glycosyltransferases appears to be the modification of secreted glycoproteins. Thus, the study of the orientation of these enzymes in the endoplasmic membranes may contribute to the knowledge of the glycosylation steps in the lumen of the endoplasmic membranes up to secretory vesicles. Studies on enzymes involved in the N-glycosylation process show an orientation of these enzymes on the cytoplasmic side of the membranes [8,9]. Enzymes involved in the last steps of glycosylation, i.e., sialyltransferase and galactosyltransferase are demonstrated to be located on the luminal side of the Golgi membranes [10–12].

The modulation of glycosyltransferases by phospholipids may reflect their affinity for their lipidic environment, a function of their position in the membranes. As many other enzymes, membrane-bound glycosyltransferases are known to require a specific phospholipid environment [13–17]. We have previously shown that a sheep brain membrane-bound fucosyltransferase [18] was activated by polar phospholipids such as lysoPA and lysoPI [19] and neutral phospholipids such as lysoPC which markedly enhanced this fucosyltransferase activity as has also been reported for other membrane-bound glycosyltransferases [20,21]. This stimulation is mediated in part through a direct interaction of lysoPC by its solubilization properties [19,22,23].

We report here a difference in behaviour of lysoPC for two brain sialyltransferases which has brought us to study the accessibility of these enzymes in the microsomal vesicles by measuring their reactivity to DIDS, a specific anion inhibitor (described by Spiro and Spiro [8] as a non-penetrating probe) and their susceptibility to trypsin.

Materials and Methods

Chemicals. All reagents were of analytical grade. Fetuin (grade III), bovine submaxillary mucin, human serotransferrin, Triton X-100 and other detergents, CMP-NeuAc, DIDS, trypsin, soybean trypsin inhibitor and phospholipids such as PE and lysoPC from egg, PI from soybean, PS from bovine brain were purchased from Sigma. Dipalmitoyl-PA and PC were from Medmark (Munich, F.R.G.). LysoPA and lysoPE palmitoyl, lysoPI from pig liver, lysoPS from bovine brain and synthetic lysoPC from Serdary Research Laboratories (London, Ontario, Canada). CMP-[^{14}C]NeuAc (spec. act. 290 Ci/mol) and UDP-[^{14}C]Gal (spec. act. 337 Ci/mol) were obtained from New England Nuclear.

Animals. Male rats, weighing from 180 to 200 g, were OFA strain (obtained from Sprague-Dawley strain) purchased from IFFA-Credo (Les Oncins, France).

Enzymatic preparation. Rats were killed by decapitation without anaesthetics. The cerebral hemispheres were removed immediately after death and homogenized in a Potter-Elvehjem-type homogenizer in 50 mM Tris-HCl buffer (pH 7.2), 330 mM sucrose (9 ml/g of wet tissue). Initial

centrifugation at $1000 \times g$ for 10 min eliminated the nuclei. The resulting supernatant was centrifuged at $20000 \times g$ for 20 min removing mitochondria, synaptosomes and lysosomes as a pellet. The centrifugation of the supernatant at $105000 \times g$ for 60 min led to the sedimentation of the microsomal fraction which was immediately frozen. Just before use, pellets were thawed and suspended in 50 mM Tris-HCl buffer (pH 7.2). The solubilization of membrane-bound sialyltransferases by Triton X-100 or by lysoPC was performed by a subsequent centrifugation of the microsomes at $150000 \times g$ for 60 min. The supernatants obtained were called 'Triton supernatant' and 'lysoPC supernatant', respectively. The sialyltransferase activities were measured in the supernatant and in the residual pellet.

Protein assay. Protein was determined according to the procedure of Lowry et al. [24] upon precipitation of protein with trichloroacetic acid (10%, w/v).

Desialylation of acceptors. The desialylation of fetuin, bovine submaxillary mucin, human serotransferrin and orosomucoid was by mild acid hydrolysis (0.05 M H_2SO_4, 80°C, 60 min) and verified by the resorcinol-HCl method [25].

Sialyltransferase assays. To 200 µl of brain microsomal suspension in 50 mM Tris-HCl buffer (pH 7.2), containing 400 µg protein, were added 400 µg exogenous acceptor (fetuin or asialofetuin), 0.5% Triton X-100, 5 mM $MnCl_2$, 40 mM Mes (pH 6.0) and 1.85 kBq CMP-[^{14}C]NeuAc. The incubations were performed for 120 min at the optimal temperatures corresponding with the enzymes. The reaction was stopped with 2 ml of a mixture of trichloroacetic acid (10%, w/v) and phosphotungstic acid (5%, w/v). The precipitate was filtered on GF-B Whatman filters as described previously [26]. Each enzymatic assay was done in duplicate.

Incubation with exogenous phospholipids. Phospholipids were dissolved in chloroform/methanol (2 : 1, v/v) and dried under nitrogen in the assay tubes prior to addition of the enzymatic preparation. Phospholipids were resuspended in a Branson Ultrasonic water-bath (Bransonic 220) for 2 × 30 s. The reagents for glycosylation were then added and [^{14}C]NeuAc incorporation was under standard conditions.

Galactosyltransferase assay. To 200 µl of brain microsomal suspension in 50 mM Tris-HCl buffer (pH 7.2), containing 400 µg protein were added 400 µg ovomucoid, 0.5% Triton X-100, 10 mM $MnCl_2$, 40 mM Mes (pH 6.0) and 1.85 kBq UDP-[^{14}C]Gal. The incubation was at 25°C for 30 min and the reaction was stopped as described above.

Analysis of the CMP-[^{14}C]NeuAc degradation. The degradation of CMP-[^{14}C]NeuAc during the incubation experiment was followed by chromatography on 3MM Whatmann paper for 16 h at pH 3.8 in 1 M sodium acetate/ethanol (30 : 70, v/v) according to Mookerjea and Yung [27].

Incubation with DIDS. To 2 ml of brain microsomal suspension in 50 mM Mes (pH 6.5), 0.25 M saccharose, DIDS was added from 0 to 60 µM. After preincubation for 10 min at 27°C as described by Spiro and Spiro [8], the suspensions were centrifuged at $150000 \times g$ for 60 min to pellet the microsomes. The supernatants, containing excess of DIDS, were discarded. The pellets were resuspended in the same volume of 50 mM Tris-HCl (pH 7.2). The sialyltransferase and galactosyltransferase activities were measured onto 200 µl of each suspension as described above. To study the effect of Triton X-100 on the DIDS inhibitory potency, brain microsomal suspensions preincubated with DIDS were directly checked for the sialyltransferase and galactosyltransferase activities without centrifugation, in the presence of 0.5% Triton X-100.

Trypsin susceptibility measurement. To 6 ml of brain microsomal suspension in 50 mM Tris-HCl (pH 7.2) at a concentration of 1–2 mg/ml, was added 60 µg of trypsin (final concentration: 10 µg/ml). The suspension was preincubated at 23°C with or without 0.5% Triton X-100. At given times, aliquots of 1.45 ml were removed from the mixture and added to 0.1 volume of soybean trypsin inhibitor (5 mg/ml). Galactosyl- and sialyltransferase activities were both assayed with 200 µl of each aliquot as described above. All assays were in the presence of Triton X-100.

Results

Evidence for two distinct brain sialyltransferases

An unusual sialyltransferase activity acting on native fetuin was previously described [6]. It was

found to be a NeuAcα(2-3)Galβ(1-3)GalNAcα(2-6)-sialyltransferase and designated below as fetuin sialyltransferase. Its temperature optimum was 28°C. This enzyme could not work on asialofetuin as the presence of NeuAc in α(2-3)-linkage to the galactose residue is an absolute requirement for this fetuin sialyltransferase activity. Another sialyltransferase, transferring NeuAc onto asialofetuin only, also was present in rat brain microsomes whereas other acceptors such as serotransferrin, orosomucoid, bovine submaxillary mucin desialylated or not, were not effective. The latter enzyme, called asialofetuin sialyltransferase, is specific for *O*-glycans. This enzyme is a Galβ(1-3)GalNAcα(2-3)-sialyltransferase, transferring NeuAc in α(2-3)-linkage to the galactose residue (unpublished results). Its temperature optimum was 20°C. Other requirements such as Mn^{2+}, pH (6.0) were the same for the two enzymes. Maximal activity was obtained with 0.5% Triton X-100 for the two enzymes. Other neutral detergents (Custcum, Emulphogen, Cemulsol NPT 12, Triton CF54, Triton WR, Nonidet) have been studied but they were less effective than Triton X-100. All acidic detergents strongly inhibited the two sialyltransferases (data not shown).

Effect of exogenous phospholipids on membrane-bound sialyltransferases

As both sialyltransferases were membrane-bound enzymes, the modulation of their activities by phospholipids was investigated. Results presented in Table I show that, in absence of detergent, the sialyltransferase activities were strongly modified by the addition of some exogenous phospholipids at 5 mM. All the acidic phospholipids (PA, lysoPA, PI, lysoPI, PS, lysoPS) inhibited the two enzymes. The neutral phospholipids (PC, PE, lysoPE) had no significant effect, except for lysoPC which slightly inhibited the asialofetuin sialyltransferase whereas the fetuin sialyltransferase was strongly activated. Glycerophosphorylcholine was

TABLE I

EFFECT OF PHOSPHOLIPIDS ON TWO BRAIN SIALYLTRANSFERASES IN ABSENCE OR PRESENCE OF TRITON X-100

Each reaction mixture contained 5 mM of lipidic compounds homogenized by sonication with microsomal suspension (containing 0.4 mg protein) as described in Materials and Methods. 0.4 mg of acceptor (fetuin or asialofetuin), 5 mM $MnCl_2$, 40 mM Mes (pH 6.0) and 1.85 kBq of CPM-[^{14}C]NeuAc were then added, with or without 0.5% Triton X-100. Incubations were performed at 20°C and 28°C, respectively, for the asialofetuin sialyltransferase and the fetuin sialyltransferase. The enzyme activity is expressed in pmol/120 min per mg protein and, in brackets, as inhibition (−) or activation (+) percentages compared to the control without lipids. The data are the means of two separate values. S.D. does not exceed 10%.

Lipid added	Enzyme activity			
	asialofetuin sialyltransferase		fetuin sialyltransferase	
	− Triton	+ Triton	− Triton	+ Triton
None	13.5 (0)	33.2 (0)	17.6 (0)	69 (0)
Glycerophosphorylcholine	1.1 (−92)	2.7 (−92)	2.5 (−86)	1.4 (−98)
Glycerophosphorylethanolamine	13.9 (+3)	31.9 (−4)	17.9 (+2)	69 (0)
Glycerophosphorylinositol	13.5 (0)	22.6 (−32)	16.4 (−7)	42.8 (−38)
Glycerophosphorylserine	11.5 (−15)	13.6 (−59)	8.1 (−54)	25.5 (−63)
Lysophosphatidylcholine	11.3 (−16)	20.7 (−38)	95 (+540)	75 (+8)
Lysophosphatidylethanolamine	13 (−4)	37 (+10)	16 (−9)	73.8 (+7)
Lysophosphatidic acid	3.7 (−73)	5 (−85)	1.2 (−93)	8 (−88)
Lysophosphatidylinositol	0.7 (−95)	1.4 (−96)	0.6 (−97)	1.4 (−98)
Lysophosphatidylserine	10.3 (−24)	20 (−39)	13.3 (−24)	50 (−28)
Phosphatidylcholine	11.9 (−12)	36 (+8)	18.2 (+3)	59 (−14)
Phosphatidylethanolamine	12.6 (−7)	34 (+2)	16.2 (−5)	66.4 (−4)
Phosphatidic acid	8.6 (−36)	3.6 (−89)	12 (−31)	14.7 (−79)
Phosphatidylinositol	10.1 (−25)	8.1 (−76)	6.1 (−65)	18 (−74)
Phosphatidylserine	10 (−25)	12.5 (−62)	11.4 (−35)	16.7 (−76)

a strong inhibitor for the two enzymes.

In presence of Triton X-100, the results are of the same type, but the stimulatory effect of the fetuin sialyltransferase by lysoPC found in absence of Triton X-100 was masked. This finding has been reported also for other glycosyltransferases [20–21,23].

Effect of lysoPC concentration on the two brain sialyltransferases

As reported above (Table I), in absence of Triton X-100, 5 mM lysoPC inhibited the asialofetuin sialyltransferase and activated the fetuin sialyltransferase. In these investigations various concentrations of lysoPC were used. As shown in Fig. 1, lysoPC strongly stimulated the fetuin sialyltransferase proportionally to concentration, up to 10 mM whereas the asialofetuin sialyltransferase was slightly activated at 1 mM and inhibited at higher concentrations.

Solubilization effect of lysoPC

The solubilization properties of lysoPC compared to 0.5% Triton X-100 was investigated for the two enzymes at their optimal effective lysoPC concentrations (i.e. 5 mM for fetuin sialyltransferase and 1 mM for asialofetuin sialyltransferase). The sialyltransferase activities were measured in each supernatant and pellet as described in Materials and Methods. 0.5% Triton X-100 was added to the pellets to measure the residual activity. A microsomal suspension centrifuged without any detergent was used as a control. Results presented in Table II show that, in the absence of detergent, the two sialyltransferase activities (98%) were recovered in the pellet and no solubilization occurred. In the presence of 0.5% Triton X-100, 78% and 83% of asialofetuin sialyltransferase and fetuin sialyltransferase, respectively, were solubilized. In the presence of 1 mM lysoPC no solubilization was found for the fetuin sialyltransferase, and the asialofetuin sialyltransferase was slightly solubilized (10% recovered in the supernatant). In the presence of 5 mM lysoPC, the fetuin sialyltransferase was almost completely solubilized. The activity recovered in the supernatant was above 100% (123%) indicating an enhancement of the fetuin sialyltransferase activity greater than that obtained in the presence of Triton X-100. For asialofetuin sialyltransferase, only 35% of the initial activity was recovered, indicating that this enzyme is strongly inhibited by 5 mM lysoPC as already shown in Table I and Fig. 1.

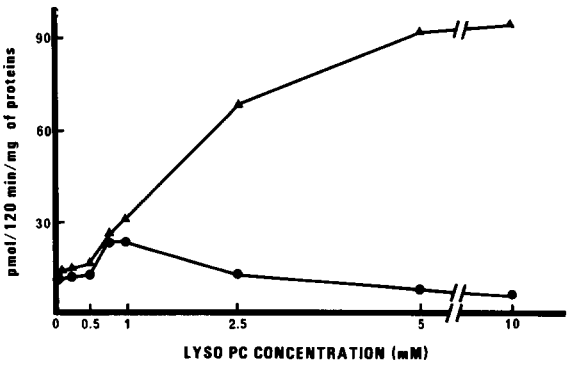

Fig. 1. Influence of lysoPC concentrations on the two brain sialyltransferase activities. Enzyme assays were performed as described in Tables I and II, without Triton X-100. Each sample was incubated in the presence of a different lysoPC concentration, from 0 to 10 mM for 120 min at 20°C for asialofetuin sialyltransferase (●———●) and at 28°C for the fetuin sialyltransferase (▲———▲).

Influence of the chain length and the degree of unsaturation of fatty acyl chains from lysoPC

The choline moiety of lysoPC appeared as an essential factor for its specific action on the two brain sialyltransferases as the other neutral lysophospholipids were without effect. But since glycerophosphorylcholine was a strong inhibitor whereas lysoPC activated the fetuin sialyltransferase, the presence of one fatty acid is an other essential factor for the modulation of the sialyltransferases. To clarify the effect of fatty acyl chains of lysoPC on the two enzymes, other studies were carried out with synthetic lysoPC having different chain lengths and degree of unsaturation of the fatty acids. Natural lysoPC from egg yolk was a mixture of palmitoyl- and stearoyl-lysoPC. Results presented in Table III show that a marked enhancement was obtained with saturated fatty acyl chains from C_{16} to C_{18} for the fetuin sialyltransferase whereas the best activity was obtained with C_{18} fatty acyl chain for the asialofetuin sialyltransferase. LysoPC comprising unsaturated fatty acyl chains decreased the sialyltransferase activities except for the $C_{18:1}$ lysoPC. The two enzymes reacted differently to $C_{18:1}$ lysoPC de-

TABLE II

SOLUBILIZATION ASSAYS FOR THE TWO BRAIN SIALYLTRANSFERASES BY lysoPC AND TRITON X-100

The solubilization of sialyltransferases was performed as described in Materials and Methods. Percent activity in assays with detergent is expressed in comparison with the control assayed without any detergent (referred as 100%).

Detergent added	Enzyme activity					
	asialofetuin sialyltransferase			fetuin sialyltransferase		
	activity in each fraction (pmol)	activity (%)		activity in each fraction (pmol)	activity (%)	
None						
Supernatant	5	3	100	4	1	100
Pellet	160	97		318	99	
0.5% Triton						
Supernatant	110	67	94	211	66	91
Pellet	44	27		80	25	
1 mM lysoPC						
Supernatant	8	5	95	4	1	101
Pellet	149	90		321	100	
5 mM lysoPC						
Supernatant	35	21	35	322	100	123
Pellet	23	14		74	23	

TABLE III

INFLUENCE OF THE LENGTH AND DEGREE OF UNSATURATION OF lysoPC FATTY ACYL CHAINS ON THE SIALYLTRANSFERASE ACTIVITIES

Incubation assays were performed as previously described. LysoPC concentration was 1 mM for the asialofetuin sialyltransferase and 1 mM (a) and 5 mM (b) for the fetuin sialyltransferase. Results are expressed in pmol/120 min per mg protein and comprise the means of two separate values. S.D. doesn't exceed 10%.

Compound	Enzyme activity		
	asialofetuin sialyltransferase	fetuin sialyltransferase	
None	12	17	
0.5% Triton X-100	35	75	
LysoPC (egg yolk)	23.5	[a] 29	[b] 100
$C_{12:0}$	11.4	21	65.2
$C_{14:0}$	11.7	30	72
$C_{16:0}$	14.4	34	106
$C_{17:0}$	18.6	47	105
$C_{18:0}$	26	40	104
$C_{18:1}$ *cis*	24.3	20	65
$C_{18:1}$ *trans*	14	33	94
$C_{18:2}$	13.8	12	19.5
$C_{18:3}$	18.5	12	11

pending on the configuration of $C_{18:1}$ which in the *cis*-configuration enhanced the asialofetuin sialyltransferase whereas in the *trans*-configuration, it was not effective. An opposite effect was found with lysoPC (at 1 mM as at 5 mM) for the fetuin sialyltransferase which is slightly stimulated by the $C_{18:1}$ *cis*-configuration while in *trans*-configuration, the activation was maximal.

Effect of the specific anion inhibitor DIDS on the two brain sialyltransferases

DIDS was described by Spiro and Spiro [8] as a non-penetrating probe which interacts with the anion binding sites (sugar nucleotide sites) of glycosyltransferases oriented closely towards the cytoplasm.

The two brain membrane-bound sialyltransferases were dependent on their hydrophobic environment to be completely active. As lysoPC, known to possess detergent properties, interferes differently with these two enzymes, we postulated that they could be oriented differently in the membrane. For this purpose, the accessibility of the two sialyltransferases to DIDS was studied. Ovomucoid galactosyltransferase was used as a

marker for the luminal side of the Golgi membrane as previously reported by Fleischer [10] and therefore was not accessible to DIDS.

The inhibitory effect of DIDS was previously controlled onto enzymes removed from their membranes after solubilization by Triton X-100. The two sialyltransferases were both inhibited 90% by DIDS (data not shown).

After preincubation of intact brain microsomes with different concentrations of DIDS and after centrifugation, sialyltransferase and galactosyltransferase activities were measured in the pellets. Table IVa shows that the ovomucoid galactosyltransferase was weakly inhibited (−24%) by 60 μM DIDS. This result suggests that the brain endoplasmic membranes were slightly damaged during DIDS treatment. Furthermore, this slight inhibition indicated that isolated endoplasmic membranes maintained the orientation that they possessed in vivo. The fetuin sialyltransferase, in contrast with the ovomucoid galactosyltransferase, was more strongly inhibited by DIDS, (up to 50% at 60 μM DIDS) while, under the same conditions, the asialofetuin sialyltransferase was only inhibited by 18%. Asialofetuin sialyltransferase and ovomucoid galactosyltransferase are both protected against DIDS inhibition in intact membranes, suggesting the same orientation, whilst fetuin sialyltransferase which is more easily accessible to DIDS, appears located closely towards the cytoplasmic side of the endoplasmic membranes.

In membranes disrupted by Triton X-100 (Table IVb), ovomucoid galactosyltransferase was inhibited up to 58% by 60 μM DIDS, demonstrating a better accessibility after disruption of membranes, and confirming the internal localization of this enzyme. The two brain sialyltransferases were also strongly inhibited by DIDS, but to an higher extent than for the ovomucoid galactosyltransferase (93% and 85% inhibition, respectively, for the fetuin sialyltransferase and the asialofetuin sialyltransferase). These results suggest that the membranes were not entirely disrupted by Triton X-100 and that DIDS proportionally inhibited the three enzymes according to their accessibility, the fetuin sialyltransferase appearing to be the more accessible and ovomucoid galactosyltransferase the least.

TABLE IV

EFFECT OF ANION SPECIFIC INHIBITOR, DIDS, ON BRAIN GALACTOSYL- AND SIALYLTRANSFERASES

Table IVa. Effects of DIDS on intact membranes. As described in Materials and Methods, a suspension of brain microsomes in 50 mM Mes (pH 6.5), 0.25 M saccharose was preincubated with 0, 20, 40 or 60 μM DIDS for 10 min at 27°C. The acellular assays were centrifuged at 150000 × *g* for 60 min to eliminate excess of DIDS. The pellets were resuspended in 50 mM Tris-HCl (pH 7.2) and the enzyme activities were determined in presence of Triton X-100, as described in Materials and Methods onto 200 μl of the suspension. Results are expressed in pmol/120 min per mg protein and, in brackets, as percentages compared to the control without DIDS (−, inhibition; +, activation). These data comprise the means of two separate values.

DIDS (μM)	Enzyme activity		
	asialofetuin sialyltransferase	fetuin sialyltransferase	ovomucoid galactosyltransferase
0	34 (0)	73 (0)	38 (0)
20	33 (−3)	51 (−30)	31 (−18)
40	33 (−3)	47 (−36)	32 (−16)
60	28 (−18)	36 (−51)	29 (−24)

Table IVb. Effect of DIDS on membranes disrupted by Triton X-100. 200 μl of brain microsomal suspension were preincubated with the different concentrations of DIDS, then all the components of the enzyme assays were added, particularly Triton X-100, and enzyme activities measured. Results are expressed as in Table IVa.

DIDS (μM)	Enzyme activity		
	asialofetuin sialyltransferase	fetuin sialyltransferase	ovomucoid galactosyltransferase
0	33 (0)	71 (0)	38 (0)
20	18 (−45)	24 (−66)	28 (−26)
40	11 (−67)	8 (−89)	23 (−39)
60	5 (−85)	5 (−93)	16 (−58)

Effect of trypsin on brain sialyltransferases in intact and in disrupted membranes

The fetuin sialyltransferase showed a better accessibility to DIDS, compared to asialofetuin sialyltransferase and ovomucoid galactosyltransferase. To confirm these results, the susceptibility of these enzymes to trypsin treatment was investigated in intact and disrupted membranes.

Table Va shows the sensitivity of sialyl- and galactosyltransferases to trypsin (10 μg/ml) in intact membranes. The results show that the

TABLE V

EFFECT OF TRYPSIN ON BRAIN SIALYLTRANSFERASES AND GALACTOSYLTRANSFERASE

Microsomal suspension in 50 mM Tris-HCl (pH 7.2) were treated with 60 μg of trypsin at 23°C in the absence (Table Va) or presence (Table Vb) of 0.5% Triton X-100. After addition of trypsin, at given times 1.45 ml were removed, 0.145 ml solution of soybean trypsin inhibitor 5 mg/ml were added and the glycosyltransferase assays were performed as described in Materials and Methods in the presence of 0.5% Triton X-100. The enzyme activity is expressed in pmol/120 min per mg protein and, in brackets, as inhibition (−) or activation (+) percents compared to the control. These data are the means of two separate values.

Table Va. Effect of trypsin on intact membranes.

Time (min)	Enzyme activity		
	asialofetuin sialyltransferase	fetuin sialyltransferase	ovomucoid galactosyltransferase
0	36 (0)	66 (0)	40 (0)
10	33 (−8)	64 (−3)	34 (−15)
20	31 (−14)	56 (−15)	32 (−20)
30	29 (−19)	58 (−12)	31 (−23)

Table Vb. Effect of trypsin on membranes disrupted by 0.5% Triton X-100.

Time (min)	Enzyme activity		
	asialofetuin sialyltransferase	fetuin sialyltransferase	ovomucoid galactosyltransferase
0	34 (0)	69 (0)	45 (0)
10	13 (−62)	72 (+4)	19 (−58)
20	7 (−80)	66 (−4)	10 (−78)
30	4 (−87)	51 (−27)	6 (−87)

asialofetuin sialyltransferase and the ovomucoid galactosyltransferase were slightly inhibited (19% and 23%, respectively). This inhibition of the ovomucoid galactosyltransferase reflected the slight damage of the endoplasmic membranes previously found with DIDS. The fetuin sialyltransferase is inhibited only by 12%

The effect of trypsin on membranes disrupted by Triton X-100 (Table Vb) shows that, as expected, the ovomucoid galactosyltransferase was strongly inhibited after 30 min of trypsin treatment (87%), confirming the internal localization of this enzyme. The same result was obtained with the asialofetuin sialyltransferase which seems, like galactosyltransferase, to be in an internal position in the membrane. The most unexpected result was that obtained with the fetuin sialyltransferase; after trypsin treatment for 30 min only 27% inhibition was found. Therefore, the fetuin sialyltransferase, for which DIDS results indicated an orientation towards the cytoplasmic side of endoplasmic membranes, was more resistant to trypsin treatment than the other enzymes even after membrane disruption by Triton X-100.

To confirm this difference in susceptibility of asialofetuin and fetuin sialyltransferases to trypsin, and to eliminate the possibility of a direct effect of trypsin on their membranous environment, the effect of trypsin was investigated on the two brain sialyltransferases after their complete solubilization by 0.5% Triton X-100 and elimination of the membranes by centrifugation (as described in Materials and Methods). Fig. 2 shows the difference of reactivity to trypsin (5 μg/ml) of the two sialyltransferases in 'Triton supernatant'. The fetuin sialyltransferase was only inhibited by 8% after 30 min of incubation with trypsin, while

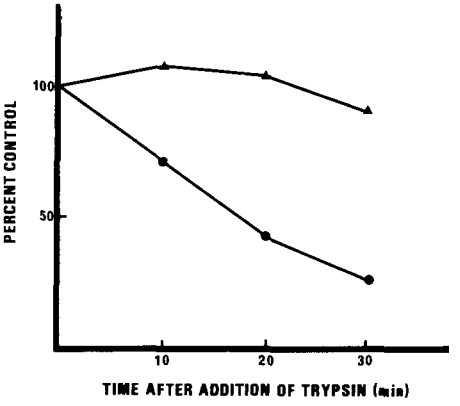

Fig. 2. Sensitivity of the two solubilized brain sialyltransferases to trypsin treatment. After solubilization of the two brain sialyltransferases by Triton X-100 (as described in Materials and Methods), 'Triton supernatant' (5 ml, 5.5 mg protein) was treated by trypsin (25 μg) at 23°C. After addition of trypsin, at given times 1.25 ml were removed, 0.125 ml solution of soybean trypsin inhibitor 5 mg/ml were added and asialofetuin sialyltransferase (●——●) and fetuin sialyltransferase (▲——▲) activities were measured in the absence of Triton X-100. Results are expressed as a percentage of the specific activity of the control where trypsin inhibitor was added with trypsin before addition of 'Triton supernatant'.

under the same conditions the asialofetuin sialyltransferase activity was decreased by 75%.

Discussion

The present report is a study about the topography in the endoplasmic membranes of two distinct rat brain sialyltransferases, acting on fetuin and on asialofetuin, respectively. We suggest that the fetuin sialyltransferase is oriented closely towards the cytoplasmic side of the membranes whereas the asialofetuin sialyltransferase shows a more internal position.

The first indication of such a difference in orientation aroused from a comparative investigation on the modulation of these two enzymes by phospholipids, providing evidence for a close relationship between lipidic environment and sialyltransferase activities. In the absence of detergent, exogenous charged phospholipids strongly inhibited these two enzymes. Neutral phospholipids were without effect except for lysoPC. At non-solubilizing concentration (1 mM), the two brain sialyltransferases were equally slightly stimulated, while at a 5 mM concentration the two enzymes behaved differently. Fetuin sialyltransferase was stimulated, as already shown for brain fucosyltransferase [19] and some other membrane-bound glycosyltransferases [20,21,28]. This stimulation is mediated in part by the solubilization properties of lysoPC, in agreement with other reports [19,22,23]. Under the same conditions, the asialofetuin sialyltransferase was inhibited.

Studies on the nature of the fatty acid moiety of lysoPC (used at 1 mM for asialofetuin sialyltransferase and 1 mM or 5 mM for fetuin sialyltransferase) have shown that the highest activity for the two enzymes was obtained with C_{16} and C_{18} lysoPC. The same results were obtained by Serres-Guillaumond et al. [19] on the rat brain fucosyltransferase, Mookerjea et al. [20] and Westcott et al. [28] on a liver microsomal galactosyltransferase. C_{18} lysoPC with one or several double bonds are less effective, except for $C_{18:1}$ lysoPC which interfered differently with the two sialyltransferases according to its *cis*- or *trans*-configuration. The fetuin sialyltransferase in the presence of 1 or 5 mM lysoPC, is stimulated by *cis*-$C_{18:1}$ and *trans*-$C_{18:1}$ lysoPC but the best activity is obtained with the *trans*-$C_{18:1}$ lysoPC. We thus conclude that the fetuin sialyltransferase does not seem to be sensitive to membrane modifications. In an opposite way, the asialofetuin sialyltransferase is only activated by *cis*-$C_{18:1}$ lysoPC. Kitagawa et al. [29] have reported an increase of membrane fluidity in the presence of *cis*-unsaturated fatty acids and no effect of *trans*-unsaturated fatty acids. Maximal enzymatic activation by 1 mM *cis*-$C_{18:1}$ lysoPC found in the present study might be explained by an increase in membrane fluidity, leading to a better accessibility for the substrate, but without enzymatic solubilization.

The fact that the sugar nucleotides are negatively charged under physiological conditions had led us to study DIDS in the same way as Spiro and Spiro [8] and Capasso and Hirschberg [30], i.e., as a specific anion inhibitor which will interfer with the anionic sugar nucleotide site. Spiro and Spiro [8] also described DIDS as a non-penetrating probe. Our investigations using DIDS, and trypsin show a difference in accessibility of the two sialyltransferases in the membrane.

The asialofetuin sialyltransferase reacted in the same way as the ovomucoid galactosyltransferase towards DIDS treatment. In the absence of detergent, these two enzymes were only slightly inhibited, while in the presence of detergent their activities were strongly decreased. Therefore, they were not easily accessible to DIDS in intact membranes and these data suggested an internal position for the asialofetuin sialyltransferase in Golgi membrane as found for the ovomucoid galactosyltransferase. Previous studies had shown a similar orientation for galactosyltransferase and sialyltransferase in rat liver [10–12].

Our data from trypsin treatment corroborated these conclusions. In the absence of detergent, asialofetuin sialyltransferase and ovomucoid galactosyltransferase were inhibited by 19 to 23%. After membrane disruption by Triton X-100, the inhibition increased up to 87%. The 20% inhibition obtained with DIDS or trypsin in the absence of detergent for the ovomucoid galactosyltransferase suggests that the membranes were only slightly damaged. Since ovomucoid galactosyltransferase was described as an enzyme oriented towards the luminal side of the Golgi membrane [10], the

polarity of the membranes is maintained.

The asialofetuin sialyltransferase possesses a strong affinity for its lipidic environment, explained by its internal position. This hypothesis is corroborated by the loss of its activity when the enzyme is removed from the membrane by action of 5 mM lysoPC, indicating a change in its enzymatic lipidic environment.

Results obtained on fetuin sialyltransferase show a better accessibility to DIDS compared to the asialofetuin sialyltransferase and the ovomucoid galactosyltransferase, suggesting an orientation closely towards the cytoplasmic side of the endoplasmic membrane. Spiro and Spiro [8] and Snider et al. [9] showed such an orientation for enzymes involved in the N-glycosylation process. However, the possibility that the fetuin sialyltransferase spans the bilayer with its active site facing the lumen cannot be ruled out. Furthermore, the inhibitory effect of DIDS on fetuin sialyltransferase could not be explained by inhibition of anion transport since the inactivation of the enzyme remained after disruption of the membranes and was also higher than that in intact membranes. Our data are in agreement with those obtained by Spiro and Spiro [8].

Results on trypsin treatment show that the membrane-bound or Triton X-100 solubilized fetuin sialyltransferase was almost insensitive to trypsin, while asialofetuin sialyltransferase and ovomucoid galactosyltransferase were strongly inhibited. As fetuin sialyltransferase was assumed to be an external enzyme, we suggest that this enzyme, less protected from an external aggression than the asialofetuin sialyltransferase by its lipidic environment, has protected its active site by molecular internalization, hence the trypsin resistance. A similar resistance to proteolysis has been observed for enzymes involved in the synthesis of GlcNAc-lipid [9] and for other microsomal enzymes [31,32].

The fetuin sialyltransferase solubilized by Triton X-100 was inhibited by DIDS while trypsin was uneffective. These results lead us to propose that the anion site (sugar nucleotide binding site) of the solubilized fetuin sialyltransferase is different from its active site, since the latter is not accessible to trypsin while the anionic site is accessible to DIDS.

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