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THE CHROMAFFIN GRANULE SURFACE

LOCALIZATION OF CARBOHYDRATE ON THE CYTOPLASMIC SURFACE OF AN INTRACELLULAR ORGANELLE

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SUMMARY

Intact chromaffin granules from bovine adrenal medulla are shown to have complex carbohydrates on their external (cytoplasmic) surface. This is demonstrated by the facts (1) that granules can be agglutinated by wheat germ agglutinin, and (2) that significant amounts of sialic acid can be removed from the granule surface with neuraminidase. Glycoproteins located in the granule membrane, and not glycolipids, are the molecules that mediate wheat germ agglutinin agglutination. The possible involvement of granule surface carbohydrate in the process of exocytosis is discussed.

INTRODUCTION

Secretion of substances from membrane-bound storage granules is generally accepted to occur via exocytosis; the fusion of these granules with the plasma membrane of the secretory cell [1]. A prerequisite first step in this process is the contact that takes place between the surfaces of the two membranes that are destined to fuse with each other. The two surfaces of importance are the cytoplasmic face of the cell membrane and the cytoplasmic face of the secretory granule. Elucidation of the mechanism of fusion necessitates an understanding of the composition and arrangement of these two surfaces.

Carbohydrates have been shown to be present in both membrane-bound and soluble proteins derived from chromaffin granules (the catecholamine-containing storage granules of adrenal medulla) [2, 3]. It is a common assumption that the carbohydrates of such intracellular membranes occur exclusively on their inner (cisternal) surface [4, 5]. This assumption is derived from the fact that carbohydrates have only been found on the extracellular face of the cell membrane. Preservation of such asymmetry would necessitate the localization of carbohydrates on the inner (cisternal) surface of intracellular organelles that derive from or are destined to fuse with

Abbreviation: CMF-PBS, calcium- and magnesium-free phosphate buffered saline

the cell surface membrane. In contrast to this notion, this study details the localization of complex carbohydrates on the outer (cytoplasmic) surface of chromaffin granules.

METHODS

Preparation of chromaffin granules and isolation of granule membranes

Chromaffin granules were isolated from fresh bovine adrenal medulla by centrifugation through barriers of 1.8 M sucrose as described by Smith and Winkler [6]. The surfaces of the resulting pink pellets of chromaffin granules were rinsed with 1.8 M sucrose and the entire pellet was resuspended in the same medium. To minimize hypotonic lysis, the granule suspensions were brought slowly to isotonicity through the slow dropwise addition of distilled water under constant stirring. Granules prepared in this manner were found by enzyme analysis to contain less than 0.1 % of the mitochondria of the crude homogenate and less than 4 % of the lysosomes. A 42 % recovery of crude homogenate catecholamines was effected.

Granules isolated through barriers of 1.8 M sucrose were rapidly homogenized in 5 mM Tris-succinate buffer, pH 5.9, to hypotonically lyse the granules. The lysates were centrifuged at 105 000 × *g* for 1 h. The resulting pellet was resuspended and washed in the above manner until supernatants contained no detectable catecholamines (3–4 washes were sufficient). Pellets were resuspended in isotonic sucrose and layered over a barrier of 1.0 M sucrose and centrifuged for 90 min at 80 000 × *g*. Membranes were collected at the interface and washed once in isotonic sucrose and once in the suspension medium used for subsequent investigations.

Agglutination assay

Owing to the small size of cnromaffin granules, it was impossible to determine the actual number of individual granules, either in a dispersed suspension or in an agglutinated clump, using the visual techniques normally employed in cellular agglutination studies. Although phase contrast microscopy enabled one to observe agglutination qualitatively, quantitation had to be carried out through a turbidimetric assay based on the differential light scattering properties of agglutinated versus dispersed granules. Procedures are detailed in the figure legends.

Glutaraldehyde fixation

Chromaffin granules. A suspension of granules (5.0 mg protein/ml) was brought to a final glutaraldehyde concentration of 0.2 % through the dropwise addition of a 25 % stock solution. Fixation was carried out under constant stirring at room temperature for 1–2 hours. Excess glutaraldehyde was inactivated and removed by dialysis against 0.1 M NH_4Cl overnight followed by dialysis against Dulbecco's calcium- and magnesium-free phosphate buffered saline (CMF-PBS) for two 5-h periods. Intact, fixed granules were recovered by centrifugation. Light microscopy using phase contrast revealed that granules were not aggregated by this procedure.

Erythrocytes. Fresh human type A erythrocytes, washed repeatedly in phosphate buffered saline were fixed in 2.5 % glutaraldehyde by the above procedure with the exception that NH_4Cl inactivation and washing were carried out by centri-

fugation rather than by dialysis. Fixed cells were stored in CMF-PBS with 1.0 mM NaN_3 and diluted accordingly prior to use. It was determined that erythrocytes that had been fixed in this manner agglutinated with wheat germ agglutinin, and to the same extent with or without the appropriate concentrations of non-ionic detergent.

Inhibition of erythrocyte agglutination

Chromaffin granule membranes suspended in CMF-PBS were solubilized by bringing the suspension to a concentration of 5.0 % in non-ionic detergent (Nonidet P-40, Shell Oil Co.). Agglutination assays were carried out in 1.2 ml stoppered conical polyethylene centrifuge tubes rotated asymmetrically for 5 min at 50 rev./min on a motor with an asymmetric cam. Assay mixtures contained glutaraldehyde-fixed erythrocytes, solubilized membranes, and wheat germ agglutinin (hapten when applicable) in CMF-PBS with a final detergent concentration of 0.5 %. Following incubation, aliquots were removed and scored for the number of single cells seen under phase contrast using a hemocytometer. Controls without lectin indicated that detergent alone neither resulted in nor inhibited agglutination.

Chemical methods

Catecholamines were assayed spectrofluorimetrically on a Perkin Elmer model MPF 2A fluorescence spectrophotometer according to the method of Bertler et al. [7]. Epinephrine and norepinephrine were used as standards. Sialic acid was determined by the thiobarbituric acid method of Warren [8]. Protein measurements were according to Lowry et al. [9], using bovine serum albumin as a standard. Hexose content of glycolipids was assayed by the anthrone procedure [10].

Glycolipids were isolated from acetylated lipid extracts of purified chromaffin granule membranes by Florisil column chromatography according to Saito and Hakomori [11]. Acetylated glycolipids were deacetylated with 0.1 % sodium methoxide in chloroform/methanol (2 : 1), dried in vacuo, and resuspended to the appropriate concentration in non-ionic detergent.

MATERIALS

Bovine adrenal glands were obtained from the local slaughter house. Wheat germ agglutinin was purified from wheat germ by affinity chromatography according to Bloch and Burger [12]. Concanavalin A was purchased from Miles, whereas all other lectins were isolated as ammonium sulfate precipitates in this laboratory by Claudia Metz. *Vibrio cholerae* neuraminidase was obtained from Serva, *Clostridium perfringens* neuraminidase was from Sigma. The chitin hydrolysate was prepared by Marianne Frei, and was chromatographically determined to consist of over 80 % dimeric and trimeric GlcNAc, Type A erythrocytes were generously donated by Marianne Grob.

RESULTS

Table I shows that a wide variety of lectins with varying hapten sugar specificities were utilized to ascertain the presence and determine the nature of accessible carbohydrate moieties on the chromaffin granule surface. It was found that wheat

TABLE I

ABILITY OF VARIOUS LECTINS TO AGGLUTINATE CHROMAFFIN GRANULES

Lectin and chromaffin granules (500 μg protein/ml) were brought to a final volume of 0.2 ml in either CMF-PBS or PBS. All assays were carried out in porcelain plates with 2 cm concave wells at room temperature. Samples were mixed every 2 min by a vortex created through a gentle stream of air delivered asymmetrically to the surface of the suspension in each well. Aliquots were removed from the incubation mixture at 20 min and viewed under phase contrast (magnification 1000×). Controls without lectin were treated identically (this procedure afforded the same results with whole cells as was seen in previous agglutination work in this laboratory).

Lectin	Sugar specificity	Concentration (μg/ml)	Agglutination*
Concanavalin A	α-Me Man, Glu	50–2000	—
Wheat germ agglutinin	$(GlcNAc)_n$	50–250	++
Caragana arborescens	GalNAc	1000–1500	—
Lens culinaris	α-Me Man	1250–2500	—
Phytohemagglutinin	GalNAc	1500–2000	—
Wisteria floribunda	GalNAc	1000–1500	—
Dolichos lab lab	α-Me Man	1500–2000	—
Soybean	GalNAc	1250–2500	—

Abbreviations: α-Me Man, α-methylmannose; Glu, glucose; $(GlcNAc)_n$, *N*-acetylglucosamine or its polymers, in this case chitin hydrolysate; GalNAc, *N*-acetylgalactosamine.

* ++, maximal agglutination; —, no agglutination was observed.

germ agglutinin was the only lectin that agglutinated these granules. Wheat germ agglutinin agglutination could be inhibited by its hapten sugars GlcNAc (50 mM) and chitin hydrolysate (150 μg/ml).

It was determined that light scattering, measured as an increase in absorbance at higher wavelengths, could be employed to kinetically assay agglutination. There are ample precedents for selecting and justifying such a method [13, 14]. The time course of agglutination at various lectin concentrations shown in Fig. 1a indicates that agglutination was essentially completed within 15 min. Such agglutination does not occur either in the absence of wheat germ agglutinin or in the presence of wheat germ agglutinin accompanied by the hapten sugar. Agglutination vs. wheat germ agglutinin concentration is shown in Fig. 1b in which the 20 min "end-point" agglutination values derived from the data presented in Fig. 1a are plotted against wheat germ agglutinin concentration. The wheat germ agglutinin concentration needed to increase agglutination to half its maximal value was 80 μg wheat germ agglutinin/ml.

Two sets of control experiments were conducted to show that agglutination was not due to the fact that wheat germ agglutinin is a basic protein [15], or that agglutination is mediated by the extrusion of carbohydrates from the granule matrix during preparation or subsequent incubations of the granules. In the first set of controls, a variety of non-lectin proteins, some even more basic than wheat germ agglutinin, were utilized as possible agglutinins. The results of such tests, summarized in Table II indicate that agglutination of chromaffin granules by wheat germ agglutinin cannot be due solely to the basic nature of wheat germ agglutinin. Polylysine, an extremely basic high molecular weight amino acid polymer known to mediate nonspecific cellular aggregation [17], did indeed agglutinate the granules. However,

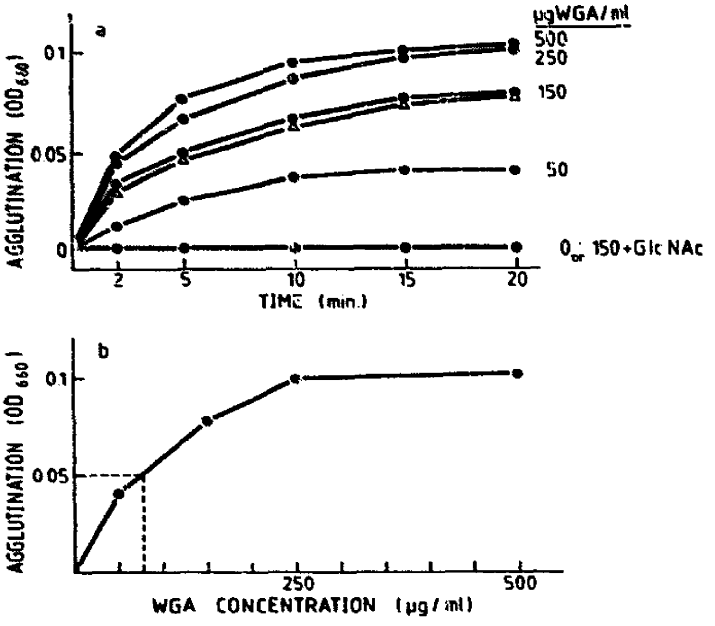

Fig. 1. Agglutination of intact chromaffin granules by wheat germ agglutinin (WGA). Turbidimetric quantitation was carried out in 0.4 ml cuvettes, with lectin (and hapten sugar when applicable) and chromaffin granules (0.1 mg protein/ml) in a final volume of 0.4 ml in CMF-PBS. The granules remained suspended in the solution due to Brownian motion, and settling of the aggregates was shown not to occur during the period of assay. (a) ● — ●, chromaffin granules; △ — △, agglutination of glutaraldehyde-fixed chromaffin granules using 150 μg wheat germ agglutinin/ml. (b) Concentration dependence of wheat germ agglutinin-induced agglutination. The 20 min end-points of (a) were replotted against wheat germ agglutinin concentration. The dashed line indicates the concentration of wheat germ agglutinin necessary for half-maximal agglutination.

TABLE II

EFFECT OF NON-LECTIN PROTEINS ON THE AGGLUTINATION OF CHROMAFFIN GRANULES

Experimental procedures are detailed in Table I.

Protein	Isoelectric point (16)	Concentration (μg/ml)	Agglutination*
Bovine serum albumin	4.9	1500	—
Cytochrome *c*	10.6	1500	—
Polylysine (mol. wt. 110 000)	10.5	500	++
Polylysine + chitin hydrolysate or α-methylmannose or glucose (50 mM)		500	++

* —, no agglutination was observed; ++, maximal agglutination.

TABLE III

NEURAMINIDASE REMOVAL OF SIALIC ACID FROM CHROMAFFIN GRANULES

Chromaffin granules (5 mg protein/ml) were incubated in either a 10% (v, v) solution of *V. cholerae* or 50 mUnits/ml *Cl. perfringens* neuraminidase for 1 h at 37 °C. The reaction mixture contained (in a final volume of 1.0 ml) 0.145 M NaCl, 5.0 mM $CaCl_2$, 2.0 mM $NaHCO_3$, enzyme solution and granules. Reactions were terminated by cooling to 0 °C, and the granules were collected by centrifugation. Supernatants were assayed for content of sialic acid as well as for catecholamines (to determine the extent of granule lysis due to incubation). Controls were incubated without enzyme and assayed identically.

	Sialic acid removed	
	(nmol/mg protein)*	% of total**
Non-enzymatic hydrolysis (0.5 M H_2SO_4, 80 °C, 1 h	46.3±3.7 (3)	
Cl. perfringens neuraminidase	22.5±1.5 (3)	49
V. cholerae neuraminidase	23.9±1.7 (3)	51

* Values are represented as the mean ±standard deviation. Numbers in parentheses indicate the number of determinations.

** Values refer to the amount of sialic acid removed enzymatically relative to the amount removed by acid hydrolysis.

there was no sugar specificity involved (in contrast to wheat germ agglutinin agglutination), as chitin hydrolysate and other lectin haptens could not inhibit this reaction. In the second set of controls designed to rule out extrusion of carbohydrate moieties from the inside of the granules, intact glutaraldehyde-fixed granules were prepared. Fig. 1a shows that these granules, which are resistant to hypotonic lysis (evidenced by lack of catecholamine release), agglutinated comparably to the unfixed granules.

A third set of controls, originally designed to further eliminate charge interactions as the cause of wheat germ agglutinin agglutination, provided strong evidence supporting localization of carbohydrates on the granule surface. Neuraminidase was used to remove the enzyme-accessible sialic acid of intact granules. Table III indicates

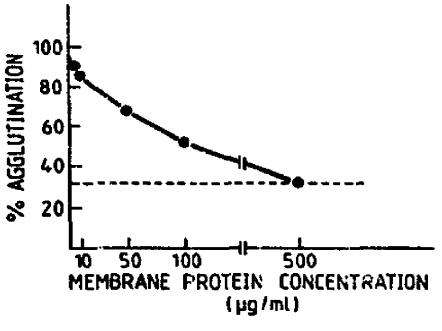

Fig. 2. Fixed erythrocyte agglutination by wheat germ agglutinin (WGA) in the presence of solubilized chromaffin granule membranes. Incubation mixtures included $1.1 \cdot 10^7$ cells/ml, 20 µg wheat germ agglutinin/ml and Nonidet P-40 at a final concentration of 0.5%. Percent agglutination is expressed as 100 (number single cells/total number cells). The dashed line refers to the level of agglutination in the absence of wheat germ agglutinin.

that fully half of the total sialic acid present in chromaffin granules is accessible to neuraminidase. Controls showed that during the enzyme treatment less than 2% of the granules' catecholamines were released; previous studies have shown that this can be accounted for by a selective loss of low molecular weight substances from the granule matrix as opposed to granule lysis [18]. Agglutination by wheat germ agglutinin of neuraminidase-treated granules, measured turbidimetrically, was reduced only 5–10% (150 μg wheat germ agglutinin/ml, 20-min incubation) refuting the notion that sialic acid on the membrane surface mediates wheat germ agglutinin agglutination [19].

Evidence proving the existence of wheat germ agglutinin receptors in the membrane is provided by experiments conducted with solubilized granule membranes and glutaraldehyde-fixed erythrocytes. Wheat germ agglutinin agglutination of fixed erythrocytes was measured in the presence of isolated chromaffin granule membranes solubilized in Nonidet P-40. Fig. 2 shows that wheat germ agglutinin specific agglutination was abolished in the presence of these solubilized membrane preparations. An identical experiment using purified membrane glycolipids indicated that the inhibition of fixed erythrocyte agglutination is due to carbohydrates on membrane proteins not on glycolipids. Amounts of chromaffin granule glycolipids corresponding to up to 10-times greater amounts of membrane than were used in the previous experiment were unable to inhibit this agglutination.

DISCUSSION

The results of this study indicate via two approaches that carbohydrates are localized on the cytoplasmic surface of chromaffin granules. Wheat germ agglutinin agglutination and the enzymatic removal of sialic acid have clearly shown that carbohydrates are accessible to these macromolecules. Agglutination by wheat germ agglutinin of both intact and glutaraldehyde-fixed granules has been shown to be specific and follow similar kinetics to those already demonstrated for this lectin in whole cell studies (Burger, M. M., unpublished). Half of the total sialic acid present in these granules is not only located on the cytoplasmic surface of the granules, but is accessible to both *V. cholerae* and *Cl. perfringens* neuraminidase. An even larger proportion of the granules' sialic acid might be on the cytoplasmic surface and merely inaccessible to the enzyme.

The presence of carbohydrates in chromaffin granules and their surface membranes is well documented [2, 3, 20, 21]. The major protein constituent of granule membranes, dopamine β-hydroxylase, is a glycoprotein whose sugar composition is known [3, 22]. The carbohydrate moiety of dopamine β-hydroxylase is composed of about one-third GlcNAc, the hapten sugar for wheat germ agglutinin, which could enable it to mediate wheat germ agglutinin agglutination. Recently, it was shown that by running large amounts of chromaffin granule membranes on acrylamide gels five bands could be stained positively for carbohydrates (periodate-Schiff procedure) [21]. This means that four other membrane glycoproteins must be considered as lectin receptors. Experiments employing surface labeling techniques are currently in progress to determine which glycoprotein(s) mediates wheat germ agglutinin agglutination.

Perhaps the most significant and controversial finding of this study is the

localization of carbohydrate chains on the external (cytoplasmic) surface of intracellular organelles. Carbohydrates have been detected in numerous intracellular membranes including nuclear envelope, endoplasmic reticulum, mitochondria, and Golgi apparatus. With the exception of mitochondria (ref. 13 and Grob, M., Fleischer S. and Burger, M. M., unpublished) and possibly nuclear envelope [23], both of which possess a double membrane, it is assumed that the carbohydrate chains are on the cisternal surfaces of these structures [5], particularly in the case of vesicles destined to fuse with the cell membrane. In chromaffin granules there is evidence suggesting that carbohydrates (concanavalin A receptors) are on the inner surface of the membrane [21]. Should this be the case, chromaffin granule membranes would be the first demonstrated instance of a membrane with carbohydrates on both surfaces.

Relevance to the process of exocytosis of accessible carbohydrate moieties on the granule surface cannot be demonstrated based upon these results. However, models can be envisioned that are consistent with these and other data based on lectin-carbohydrate interactions. Recent investigations on a variety of experimental systems have shown surface-localized carbohydrate-containing molecules mediating cell-cell recognition preceeding aggregation [24–26] and/or mating [27] phenomena. The surface localized carbohydrates of chromaffin granules in such a model would act as substrates for plasma membrane carbohydrate-binding molecules. Increasing evidence demonstrating the presence of such "lectin-like" proteins in cell surface membranes is accumulating [26, 28, 29]. In both plant and animal cells, where a specific sugar binding function has been examined, cell surface proteins have been found that possess the required specificity. The binding of the granule to the cell membrane would orient it in such a way enabling the lipid portions of the membrane to come into contact and fusion would occur as a result of the high lysolecithin (up to 25% of total phospholipid (ref. 30 and Meyer, D. I., unpublished)) content of the granule membrane. Preliminary results indicate that fusion can occur between chromaffin granules and cell surface membranes where wheat germ agglutinin can be used as a ligand.

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