Eur Biophys J (1987) 15: 141 – 145

European Biophysics Journal

Rapid lateral diffusion of lectin-labelled glycoconjugates in the human colonic adenocarcinoma cell line HT29

Comparison with the synthetic lipid analogue diI-C_{14}

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Received December 18, 1986/Accepted April 15, 1987

Abstract. The lateral diffusion of lectin-labelled glycoconjugates was studied in the human colon carcinoma cell line HT29 using fluorescence photobleaching techniques. HT29 cells were grown in either Dulbecco's modified Eagle's medium with glucose (25 m*M*; DMEM-Glu) or with galactose (25 m*M*; DMEM-Gal). Cell cultivation in the DMEM-Gal medium was assumed to promote a transformation of the cells to become small-intestinal-like with characteristic microvilli and associated enzymes. The diffusion of glycoconjugates labelled with fluoresceinated *Triticum vulgaris* agglutinin (Wheat germ agglutinin; WGA), *Ricinus communis* agglutinin-I (RCA-I), *Concanavalia ensiformis* agglutinin (ConA), *Ulex europaeus* agglutinin-I (UEA-I) and *Arachis hypogaea* agglutinin (PNA) was in all cases rapid, with a diffusion constant (D) ranging between 0.4 and 0.8×10^{-8} cm^2 s^{-1}. As a comparison the diffusion of the fluorescent synthetic lipid analog diI-C_{14} was characterized by $D = 0.8 - 1.0 \times 10^{-8}$ cm^2 s^{-1}. The diffusion of lectin-labelled surface components could not be related to the presence of microvilli on HT29 cells grown in DMEM-Gal, which ought to yield an apparently lower diffusion rate. The results indicate either that surface glycoconjugates in HT29 cells are dominated by glycolipid, or that the labelled glycoproteins are more or less free to diffuse in the plane of the membrane.

Key words: Lectins, diI-C_{14}, lateral diffusion, HT29 cells, fluorescence photobleaching

Introduction

Glycoconjugates in cell membranes play an important role in the recognition between cells and particles (Lis and Sharon 1984; Magnusson et al. 1986), and the degree of mobility of the glycoconjugates determines, for instance, how rapidly receptor molecules can be aggregated or recruited to a specific site on the cell. Rather few studies have been done to elucidate the mobility of glycoconjugates in the apical membrane of intestinal cells, but Dragsten et al. (1981) have reported that cell-surface sites labelled with fluorescently conjugated lectins (wheat germ agglutinin (WGA) and peanut agglutinin (PNA) were completely immobile. In contrast, Jacobson et al. (1976) showed that WGA-binding sites on fibroblasts had a diffusion coefficient ranging between about 2×10^{-11} and 2×10^{-10} cm^2 s^{-1}. Moreover, the lateral diffusion of lipid probes introduced into the apical membrane of the kidney epithelial cell line A6 varied between 0.29 and 1.33×10^{-8} cm^2 s^{-1}. For the synthetic lipid diI-C_{14}, i.e. ditetradecyl-indocarbocyanine, it was 0.97×10^{-8} cm^2 s^{-1} in the apical and 1.9×10^{-8} cm^2 s^{-1} in the basolateral side of the cells. In general, the diffusion rate was smaller in the apical than in the basolateral side, which was assumed to be due to the presence of microvilli on the apical side (Dragsten et al. 1981). Such a reduction can also be anticipated from a theoretical point of view (Aizenbud and Gershon 1984). More recently, however, van Meer et al. (1985) found that Rhodamine-labelled phosphatidyl ethanolamine had a varying diffusion coefficient (at 5 °C) in Madin-Darby canine kidney (MDCK) cells and Baby hamster kidney (BHK) cells, i.e. 0.69 and 2.1×10^{-9} cm^2 s^{-1}. These diffusion coefficients were, however, about ten-fold slower than those observed by Dragsten et al. (1981). The aims of our study were: First, to reevaluate glycoconjugate diffusion in cultured epithelial cells, and second to assess whether the measured diffusion coefficient of lectin-labelled glycoconjugates was influenced by the presence of microvilli on the cell surface. For this purpose, we used a human colon carcinoma cell line HT29 (Fogh

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and Trempe 1975) which can be induced to become small-intestinal-like with increased number of microvilly, as evidenced morphologically and biochemically by increased specific activity of brush-border enzymes (Pinto et al. 1982; Zweibaum et al. 1985).

Materials and methods

Cell cultures

The colon carcinoma cell line HT29 was obtained from Dr. Jorgen Fogh (Memorial Sloan-Kettering Cancer Center, Human Tumor Cell Laboratory, Rye, NY). It was grown for 14–21 days in either Dulbecco's modified Eagle's Medium with 25 m*M* glucose (DMEM-Glu) or the same medium with glucose exchanged with 25 m*M* galactose (DMEM-Gal), 10% fetal calf serum, and standard concentrations of glutamic acid, penicillin (100 µg/ml) and streptomycin (100 µg/ml). The cells were grown in DMEM-Gal on glass cover-slips for 14–21 days to allow development of small-intestine-like properties of cells (Pinto et al. 1982; Zweibaum et al. 1985).

Labelling with lectins

A coverslip with cells was rinsed by immersion × 3 into phosphate buffered saline, pH 7.3 (PBS). They were labelled at room temperature for 2 min with lectin solution (10 µg/ml in PBS), washed × 3 in PBS, and mounted right side up in a wet chamber. This was formed by putting strips of Parafilm "M"® cover paper at the edges of the coverslip with cells, a few drops (50–100 µl) of Krebs-Ringer phosphate buffer with 10 m*M* glucose, and 1 m*M* Ca^{2+} and Mg^{2+}, pH 7.3 (KRG), and another cover-slip which was sealed with a hot mixture of wax and vaseline. The experiment was started immediately and discontinued within 60 min.

To assess the effect of anti-WGA antibodies on the diffusion of WGA-labelled glycoconjugates, the WGA-antibody preparation (E.Y. Labs., San Mateo, CA) was diluted 1:100 in KRG and then applied to the washed cover-slip with lectin-labelled cells before the chamber was sealed.

Labelling with synthetic lipid

The labelling with the fluorescent lipid analog diI-C_{14} (3) (ditetradecyl indocarbocyanine; a kind gift of Dr. A. S. Waggoner, Pittsburgh, PA) was done by adding 20 µl of diI-stock solution (100 µg/ml in 99.5% ethanol) to 2 ml KRG with the coverslip with cells for 5 min at room temperature. This yields 1 µg/ml dye and 1% ethanol in the incubation mixture. The cells were then washed × 3 in KRG and a wet chamber prepared as described for lectin-labelled cells.

Lectins

The lectins were obtained either from E.Y. Labs., Inc. (San Mateo, CA) or Vector Labs. (Burlingame, CA). The following lectins were used; the carbohydrate specificity is shown in paranthesis: WGA; Wheat germ agglutinin (a.) from *Triticum vulgaris* (GluNAc (sialic acid)), RCA-I; *Ricinus communis* a. (β-D-Gal), ConA; *Concanavalia ensiformis* a. (α-D-Man, α-D-Glu), UEA-I; *Ulex europaeus* a. (α-L-fucose), and PNA; *Arachis hypogaea* a. (D-Gal-β-(1-3)-GalNAc).

Fluorescence photobleaching

The equipment used to study lateral diffusion of fluorescent membrane molecules will be described in detail elsewhere. Briefly, data aquisition and handling is done with small computers, and the mode of measurement chosen from a menu allowing:

(a) CFM, i.e. continuous fluorescence microphotolysis according to Peters et al. (1981) and Peters (1981); the computer program for evaluation of CFM was kindly given to us by Dr. R. Peters and Dr. K. Schulten.
(b) FRAP, i.e. fluorescence recovery after photobleaching, according to Peters et al. (1974), Axelrod et al. (1976), and Jacobson et al. (1976), with open measurement shutter and sampling at requested times (time resolution about 20 ms) during recovery to assess rapid diffusion, for instance, of lipids.
(c) SLOW-FRAP (Δt-variable), i.e. FRAP of slowly diffusing species, e.g. proteins, allowing measurement at gradually increasing time intervals (Δt).
(d) SLOW-FRAP (Δt-constant), i.e. as (c) but Δt set at fixed time intervals.
(e) Rapid-FRAP, i.e. FRAP but sampling of data points (< 500) is done as rapidly as is allowed by the computer (> 12 ms).

A cell sample is illuminated and bleached through a fixed, exchangeable slit, in epifluorescence through a Zeiss Universal microscope, equipped for fluorescein activation. Using a × 63 objective and a round, 160 µm diameter slit, the $1/e^2$ radius (w) was estimated as 0.89 µm. It was deter-

mined in the following ways: *(1)* Small fluorescent latex beads (0.1 μm diameter) were scanned through the illuminated spot, and the fluorescence intensity recorded with the microfluorometer, *(2)* a thin film of collodium (250 μl) with embedded diI-C_{14} (50 μl; 1 mg/ml) was prepared between a cover-slip and a microscope slide (personal communication with K. A. Jacobson, UNC at Chapel Hill, NC, as originally designed by M. Schneider, Ithaca, NY). The fluorescence from the illuminated spot was determined with a video camera connected to a digital image analysis system (3PC, Innovativ Vision AB, Linköping, Sweden). The resolution was about 0.1 μm per pixel, using the ×63 objective, and *(3)* it was calculated from the size of the aperture in the object, assuming Fraunhofer diffraction for the incident light in the aperture. *(4)* We also repeated a previous experiment with differentiated promyelocytic HL60 cells (Magnusson et al. 1983) done with FRAP-equipment in Dr. K. A. Jacobson's laboratory, UNC at Chapel Hill, NC). The lateral diffusion of WGA-labelled glycoconjugates was consistent with a value of $D = 1.0 \times 10^{-10}\,cm^2\,s^{-1}$ in both cases. This indicates that there was not any large systematic error in the calculation of the size of the illuminated spot.

The diffusion constants and percent recovery after photobleaching were calculated according to Axelrod et al. (1976) and Jacobson et al. (1976).

The laser used was a Spectra-Physics Argon laser (Type 2020-03, Spectra-Physics, Mountain View, CA) run in current mode at 20 amps, and at the 488 nm-line.

In the present investigation, the FRAP protocols described under points (b) and (e) were primarily used, since preliminary investigations had shown that the fluorescence recovery of lectin-labelled glycoconjugates was rapid. The recovery was sampled every 50 ms during 4 s with open illumination shutter, and the final point, F_∞, taken after ≧ 15 s.

Results

A. Lateral diffusion of fluorescent lectins

The lateral diffusion of lectin-labelled glycoconjugates as obtained according to the FRAP-protocol (see Materials and methods) is shown in Table 1. All the lectins diffuse rapidly with D of about $0.4-0.8 \times 10^{-8}\,cm^2\,s^{-1}$. The percent recovery varies between 50 and 80%. A representative curve for the recovery of WGA in HT29 cells grown in DMEM-Glu is shown in Fig. 1. The recovery is consistent with $D = 0.70 \times 10^{-8}\,cm^2\,s^{-1}$, and $R = 67\%$. To assess whether there was a slow diffusing component, the recovery was also measured according to the SLOW-FRAP (Δt-constant = 10 s) protocol with the final point (F_∞) taken after 200 s. In the cells tested ($n = 8$) there was no significant recovery after 10–15 s. The fluorescence had reached 90% and 97% of F_∞ after 10 and 15 s, respectively. There is no significant difference between the lectins, al though the mean D-values for WGA and ConA fall in the upper range, around $0.8 \times 10^{-8}\,cm^2\,s^{-1}$. Neither is there any clear effect of the growth condi-

Table 1. Lateral diffusion of fluorescent lectins in the human colon carcinoma cell line HT29 grown in DMEM-Gal or DMEM-Glu for 14–21 days

Lectin	DMEM-medium	$D \times 10^8$ ($cm^2\,s^{-1}$)	R (%)	n[a]
WGA	Gal	0.79 ± 0.08[b]	60 ± 2	16
	Glu	0.80 ± 0.06	59 ± 2	15
S-WGA[c]	Gal	0.48 ± 0.07	63 ± 3	8
	Glu	0.56 ± 0.08	66 ± 3	16
ConA	Gal	0.61 ± 0.03	50 ± 3	11
	Glu	0.59 ± 0.18	49 ± 2	6
S-ConA	Gal	0.48 ± 0.07	58 ± 3	5
	Glu	0.40 ± 0.06	47 ± 7	16
RCA-I	Gal	0.82 ± 0.06	49 ± 3	9
	Glu	0.56 ± 0.04	62 ± 4	8
UEA-I	Gal	0.51 ± 0.06	77 ± 2	8
	Glu	0.46 ± 0.02	79 ± 2	5
PNA	Gal	0.68 ± 0.02	68 ± 8	4
	Glu	0.56 ± 0.08	51 ± 2	5

[a] Number of cells from 2 or more samples
[b] ± Standard Error of the Mean, SEM
[c] S = Succinylated

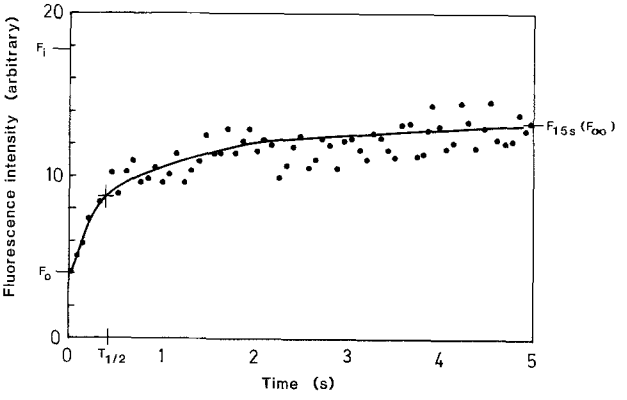

Fig. 1. Fluorescence recovery after photobleaching of fluoresceinated WGA in HT29 cells grown in DMEM-Glu; F_i = initial fluorescence before bleaching, F_0 = fluorescence after bleaching, F_{15s} = fluorescence after 15 s-recovery, approximating F_∞, i.e. maximum fluorescence recovery. Percent recovery (R) = 67%, and diffusion constant (D) = 0.7×10^{-8} $cm^2\,s^{-1}$

tions, which were assumed to promote increased formation of microvilli in HT29 cells grown in DMEM-Gal compared to cells grown in DMEM-Glu.

When anti-WGA antibodies were added to cells labelled with fluorescent WGA, two effects were seen: a slower rate of recovery and a decreased mobile fraction of labelled glycoconjugates. For instance, in one typical experiment there was no recovery in 3 out of 7 cells. Moreover, in the remaining cells the percentage recovery was reduced to much below 50%, and the diffusion coefficient varied between 0 and 0.86×10^{-8} cm^2 s^{-1}.

B. Lateral diffusion of fluorescent diI-C_{14}

After ethanol-injection of diI, the cells retained their microscope appearance and were clearly stained. According to the FRAP procedure, the diI diffusion was characterized by a D-value of $0.9 \pm 0.1 \times 10^{-8}$ cm^2 s^{-1} and an R-value of $66 \pm 11\%$. When CFM was used, after rapid pre-bleaching to extinguish immobile fluorophores, D varied between $0.8-1.0 \times 10^{-8}$ cm^2 s^{-1}.

Discussion

The present investigation aimed at addressing two questions; are lectin-labelled glycoconjugates mobile in the plane of the membrane of epithelial cells, and do microvilli affect the measured mobility? We can now conclude that lectin binding sites diffuse *rapidly* (Table 1; Fig. 1) in the apical portion of HT29 cells, in contrast to kidney epithelial cells (Dragsten et al. 1981), but the diffusion coefficient is slightly slower than for diI-C_{14}, which was $0.8-1.0 \times 10^{-8}$ cm^2 s^{-1}. Incidentally, this was equal to the value obtained by Dragsten et al. (1981) for diI-diffusion in the apical membrane of the kidney epithelium cell line A6. We can only speculate about this difference. Either the cell lines are really differnt, or did Dragsten et al. (1981) overlook a rapid fluorescence recovery, when a slow recovery can be expected for lectin-labelled entities, as observed in fibroblasts (Jacobson et al. 1976).

The incomplete recovery of diI-C_{14} (around 66%) is unexpected for a lipid probe, but could be due to adsorption, but not fusion, of lipid aggregates (vesicles), which should display little recovery (Szoka et al. 1980).

The effect of microvilli (Aizenbud and Gershon 1982, 1984) on the apparent diffusion rate cannot be confirmed in this study. Either there is no such dependence, or otherwise the combination of lectin-diffusion, and undifferentiated-differentiated cells is not a proper choice of model. In preliminary experiments we have, however, found such a dependence of microvilli in HT29 cells on the diffusion of cells labelled with fluorescent cholera toxin (unpublished observation). Therefore, lectins may be unsuitable as probes. Another reason could be that WGA and ConA cross-linked adjacent microvilli and affected the structure of the microvilli. In recent studies on the effect of ConA and WGA on small intestinal structure and permeability we have observed that the microvilli indeed become cross-linked, and furthermore club-like, as if the microfilaments within the microvilli had collapsed (Sjölander et al. 1984, 1986). The reason for the differences between WGA and S-WGA, and ConA and S-ConA (Table 1) could then be that WGA and ConA destroy the microvilli on HT29 cells, whether they are grown in DMEM-Gal or DMEM-Glu, and that results in apparently higher diffusion coefficients.

The reason for the rapid diffusion of lectin binding sites has to be further elucidated by comparison with other polarized epithelial cells. Incidentally, Dragsten et al. (1981) reported that the diffusion of lectin-labelled surface components was negligible in epithelial cells of kidney origin as assessed with fluorescence photobleaching recovery, but still the lectins somehow showed redistribution over the cell surface, which could no be explained in that study.

In the preliminary study we found that anti-WGA antibodies (E.Y. Labs, San Mateo, CA) immediately altered the recovery after photobleaching, indicating that WGA was localized to the outer part of the cell. Studies are also under way to delineate whether the glycoconjugates accessible for the lectins are lipid-bound or glycoproteins free to diffuse on the plane of the membrane.

In conclusions, we have found that lectin-labelled glycoconjugates diffuse rapidly in the apical membrane of the intestinal HT29 cells, which indicates that either the diffusing compound is a lipid (or lipid-linked) or a protein with small lateral restraints on the mobility.

Acknowledgements. Gunnel Karlsson is gratefully thanked for technical assistance with the cell cultures. This research was supported by the Swedish Medical Research Council (Project No. 6251), Erna and Victor Hasselblad Foundation, Magn. Bergvalls Stiftelse, Stiftelsen Lars Hiertas Minne, King Gustaf the Vth 80-year Fund, Östergötlands Läns Landstings Forskningsfond and The Medical Research Council of the Swedish Life Insurance Companies.

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