Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 856-863

HEADGROUP DYNAMICS OF AN INTEGRAL MEMBRANE GLYCOPROTEIN

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Received August 24,1979

SUMMARY: Glycophorin, an integral membrane glycoprotein known to be a receptor for several lectins, has been spin labelled specifically on headgroup terminal sugars. The labelled derivative has been studied in solution and also in various model membranes in an attempt to determine the factors which control headgroup dynamics. Under conditions which mimic those in a living cell the oligosaccharide chains show a uniform, relatively high freedom of motion, with individual sugar correlation times on the order of 6×10^{-10} sec to 8×10^{-10} sec depending upon the extent of glycoprotein headgroup involvement with other glycocalyx components. They exhibit no detectable occupancy of lipid or protein hydrophobic domains. Oligosaccharide dynamics are insensitive to factors which act upon that portion of the polypeptide backbone which inserts into the membrane, however a specific recognition event markedly reduces terminal sugar mobility.

INTRODUCTION

Complex contact events occurring at the eucaryotic cell surface are thought to be mediated by glycoproteins and glycolipids. Not only do these membrane components provide for the specificity of a given contact event, but it seems likely that the initial recognition process involving their oligosaccharide headgroups is followed by surface rearrangements which relate to, or even induce, the eventual cellular response (1,2). The chemical nature of oligosaccharide headgroups has been a subject of considerable interest in recent years (3-5) and, in the case of glycolipids, has been extensively catalogued. The question of headgroup dynamics has also been addressed in the case of glycolipids (6,7). However virtually nothing is known about the dynamics of membrane glycoprotein oligosaccharide moieties at the cell surface, and how such moieties respond to specific recognition events or membrane-based changes. We report here some answers to these questions obtained using the first oligosaccharide spin labelled integral membrane glycoprotein (glycophorin).

Abbreviations: PBS, phosphate buffered saline; WGA, wheat germ agglutinin

0006-291X/79/190856-08$01.00/0

MATERIALS AND METHODS

Glycophorin was isolated by the method of Marchesi and Andrews (8) and extensively ethanol washed. The glycoprotein was spin labelled as follows. 5 mg of glycophorin was dissolved in 5 ml of 0.1 M sodium acetate buffer pH 5.6 containing 0.15 M NaCl. To this was added 3.5 ml of 0.012 M sodium periodate. After 10 min at 0°C the reaction was stopped by addition of excess ethylene glycol. The solution was dialysed overnight at 4°C against 0.05 M phosphate buffer pH 7.4 containing 0.15 M NaCl, and subsequently for 2 days against distilled water containing 0.025% NaN_3. The sample was concentrated to 2.5 ml and combined with 7.5 ml of 0.2 M Na_2CO_3 buffer pH 9.5 containing 10% DMSO and 16 mg of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (Aldrich). After stirring at 0°C for 90 min, 0.5 ml of 0.6 M $NaBH_4$ in 0.01 N NaOH was added and stirring was continued for 30 min. The product was dialysed for 3 days against distilled water containing 0.025% NaN_3 and concentrated to 1.5 ml. This sample was applied to a Sephadex G50 column (0.9 x 30 cm) and eluted with 10 mM phosphate buffer pH 7.0. Spin labelled glycophorin eluted in the void volume. The spin labelled derivative migrated on SDS gels in a manner indistinguishable from unlabelled glycophorin. The number of spin labels incorporated per glycoprotein molecule was determined by double integration of the EPR spectra (less than one label per glycophorin). The product was stored frozen at -20°C.

EPR spectra were run at 21°C on a Varian E12 spectrometer interfaced with a Nicolet 1180 computer.

Two different methods were employed for incorporating labelled glycophorin into lipid bilayer systems: dialytic removal of detergent from solutions containing lipid and glycoprotein (9), and hydration of films dried down from organic solvent (10). The spectra were the same in both cases.

RESULTS AND DISCUSSION

The advantage to working with purified membrane components lies in being able to focus with minimal ambiguity on a particular system, and the human erythrocyte glycoprotein, glycophorin, is almost uniquely well suited to such an approach. Its backbone is a single polypeptide chain which traverses the membrane - the amino end bearing some 16 oligosaccharide chains with terminal N-acetylneuraminic acid residues (11-13). Furthermore it is isolable in highly pure form and may be subsequently reassembled into lipid bilayer model membranes whose recognition properties mimic those of the intact cell (9,10,14). The reaction sequence outlined in the Materials and Methods section, which can link spin labels to glycophorin N-acetylneuraminic acid residues is an adaptation of a method commonly employed to tritiate glycolipids and glycoproteins (15). Mild treatment with aqueous periodate selectively oxidizes glyceryl side chains of N-acetylneuraminic acid residues to aldehydes. These may then be reductively aminated (16) with a small amino-substituted spin label in the presence of sodium borohydride (and the unreacted side chains converted back to alcohols). In this case the spin label ring by virtue of its size and attachment (Figure 1) should mimic the behaviour of a terminal sugar

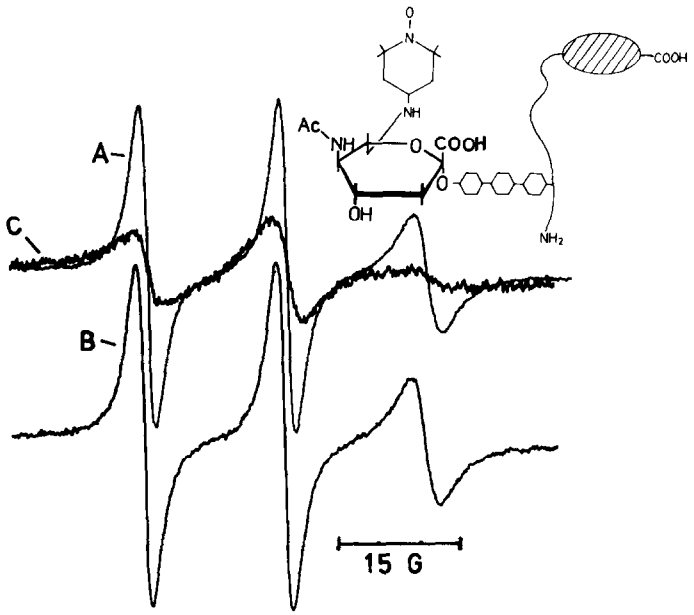

Figure 1 EPR spectra of spin labelled glycophorin
A) free in solution (PBS pH 7.5)
B) same as (A) but incorporated into unsonicated egg phosphatidylcholine bilayers
C) same as (A) but with 0.06 mM WGA
The drawing in the upper right hand corner is a representation of the labelled glycoprotein showing one hypothetical oligosaccharide chain with modified N-acetylneuraminic acid. The cross-hatched oval represents the transmembrane portion of the polypeptide backbone. All spectra recorded at 21°C.

residue in the glycoprotein headgroup. It seems likely that labels are randomly distributed amongst the various possible oligosaccharide chains. Representative EPR spectra of the labelled derivative are shown in Figure 1.

Parameters that may be derived from analysis of the EPR spectra are the correlation time, τ_c, which is inversely related to spin label mobility; and the spectral splitting, a_N, which is characteristic of the polarity of the nitroxide environment (17). We have used the expression,

$$\tau_c = 6.5 \times 10^{-10} W_o [(h_o/h_{-1})^{1/2} - 1] \quad (18)$$

to calculate correlation time, where W_o is the line width of the mid-field line, and h_o and h_{-1} are the heights of the mid-field and high-field lines respectively. Table 1 contains a compilation of these parameters derived from a variety of samples. Examination of this data shows that one facet of glycoprotein headgroup oligosaccharide behaviour which remains constant under all conditions tested to date is the highly polar nature of the spin label (and hence terminal sugar) environment. Whether free in dilute

solution, incorporated into lipid bilayers, or subject to massive headgroup crowding, the value of a_N remains within experimental error of that for a totally aqueous environment (16.8 G for water vs 14.5 G for a hydrophobic environment). When bound by WGA the terminal sugars are exposed to a somewhat more polar environment, perhaps as a result of clustering of N-acetylneuraminic acid residues. There is no evidence of oligosaccharide interaction with hydrophobic domains of lipid or protein. This is consistent with a strong H-bonding involvement of headgroup sugars. Another constant observable, but one that is not easily tabulated, is exemplified by Figure 1 A,B: the spectra are relatively homogeneous. That is they do not appear to be simple sums of several different spectra as one would expect if the headgroup contained oligosaccharide regions with very different behaviour. Hence, assuming a more or less random distribution of label amongst the 16 different oligosaccharide chains, it would appear that the majority of them behave similarly over a wide range of conditions.

In the eucaryotic cell, transmembrane glycoproteins are subject to constraints which may be intimately involved with their role at the cell surface. Firstly a portion of the polypeptide backbone is inserted into the membrane and so has an annulus of lipid. And secondly the headgroup is known to be part of a thick carbohydrate-rich layer consisting of other headgroups and adsorbed serum proteins. We have attempted to superimpose such additional considerations one by one upon the basic system of glycoprotein dissolved in buffer. We have used techniques devised by ourselves (9) and by others (10) to incorporate glycophorin into various lipid bilayer structures. Furthermore we have incubated these structures under conditions known to coat them with major glycocalyx components. The effects upon oligosaccharide spin labelled glycophorin spectral parameters are shown in Table 1. Note first of all that incorporation of glycophorin into lipid bilayers has no appreciable effect on the mobility of terminal sugars as measured by τ_c. This observation is true whether the lipid is fluid (egg phosphatidylcholine), semi-rigid (extracted erythrocyte lipid), rigid (dipalmitoyl phosphatidylcholine), or negatively charged (phosphatidylserine). Apparently, rather large alterations in the immediate environment of that portion of the polypeptide backbone which inserts into the membrane are not reflected in the dynamics of the oligosaccharide chains. We have also included in Table 1 the value of τ_c for the terminal sugar residues of membrane-incorporated gangliosides spin labelled by precisely the same procedure used on glycophorin (Lee and Grant, unpublished data). In spite of the relative complexity of the glycophorin headgroup and the fact that the ganglioside oligosaccharide chain is very similar to an individual

Table 1 Values of the correlation time, τ_c, and hyperfine splitting, a_N, derived from spectra of glycophorin spin labelled on N-acetylneuraminic acid residues. Samples were designed to test the effect of circumstances known to exist at the cell membrane.

Sample	τ_c † ($\pm$ 0.5 x 10^{-10} sec)	a_N † ($\pm$ .05 G)
labelled glycophorin free in solution (0.6 mg/ml)	9.6*	16.75
" " " + 68.5 mg/ml unlabelled glycophorin	11.3	16.75
" " " + 100 mg/ml BSA	9.6	16.75
" " " + 4 mg/ml WGA	47.7	17.00
labelled glycophorin in bilayers of egg phosphatidylcholine	10.0*	16.75
" " " " " dimyristoyl phosphatidylcholine	9.9	16.75
" " " " " extracted erythrocyte lipid	9.8	16.75
" " " " " dipalmitoyl phosphatidylcholine	10.3	16.75
" " " " " phosphatidyl serine	10.0¶	16.75
" " " " " egg phosphatidylcholine coated with Dextran T 500 (40 mg/ml) and human γ-globulin (30 mg/ml)	11.2	16.75
" " " " " egg phosphatidylcholine coated with Dextran T 500 (40 mg/ml) human γ-globulin (30 mg/ml) and BSA (40 mg/ml)	12.1	16.75
Free spin label (tempoamine) in buffer	$\sim$0.1	16.80
" " " " in hexane	$\sim$0.1	14.50
ganglioside (from beef brain) spin labelled on N-acetylneuraminic acid residues and incorporated into bilayers of egg phosphatidylcholine	13.0	16.75

†For convenience of comparison values reported are for samples run at 21°C.

*Raising the temperature to 37°C reduced these values by 1/3 to range between 6 x 10^{-10} and 8 x 10^{-10} sec.

¶For this particular sample divalent cations were exluded by the presence of 10 mM EDTA. All other samples had an aqueous phase of 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 100 mM KCl buffered at pH 7.4 with 5 mM HEPES.

glycophorin oligosaccharide chain, the ganglioside sugar residues are less mobile. The above observations are adequately explained by a model which assigns considerable flexibility to the headgroup region of the glycophorin polypeptide backbone.

Interactions which the glycoprotein headgroup would be expected to encounter in the presence of other surface components (i.e. a glycocalyx) do produce significant effects on the oligosaccharide chain dynamics. For instance when the concentration of glycophorin is raised to 69.1 mg/ml with unlabelled glycophorin in an attempt to mimic the high local headgroup densities found at the cell surface, terminal sugar correlation time is

reduced by 20%. As indicated in Table 1 a similar degree of oligosaccharide mobility reduction is seen when the lipid bilayer surface is coated (19) with serum albumin (40 mg/ml in normal plasma), γ-globulins (30 mg/ml in normal plasma), or dextran (to mimic cell surface oligosaccharide). Raising the sample temperature to 37°C has the effect (see footnote designated, *, in Table 1) of decreasing τ_c by some 33%.

Glycophorin is known to be the human erythrocyte WGA receptor, with terminal N-acetylneuraminic acid residues being implicated as binding sites for the event (20). The advantage to studying a headgroup spin labelled derivative is that one can follow the course of the WGA-glycophorin interaction, and also gain some insight into the dynamic effect of lectin binding to receptor glycoproteins in general. As illustrated in Figure 1C, binding of WGA to glycophorin induces a considerable spectral change in the direction of reduced headgroup mobility. This effect is specific and is reversed by the inhibitory sugar, N-acetylglucosamine (Table 1). Note that even very high concentrations of dextran, serum albumin, or non-specific γ-globulins fail to produce such a dramatic reduction in oligosaccharide mobility. In Figure 2 the parameter, τ_c, which is directly related to oligosaccharide immobilization is shown plotted against con-

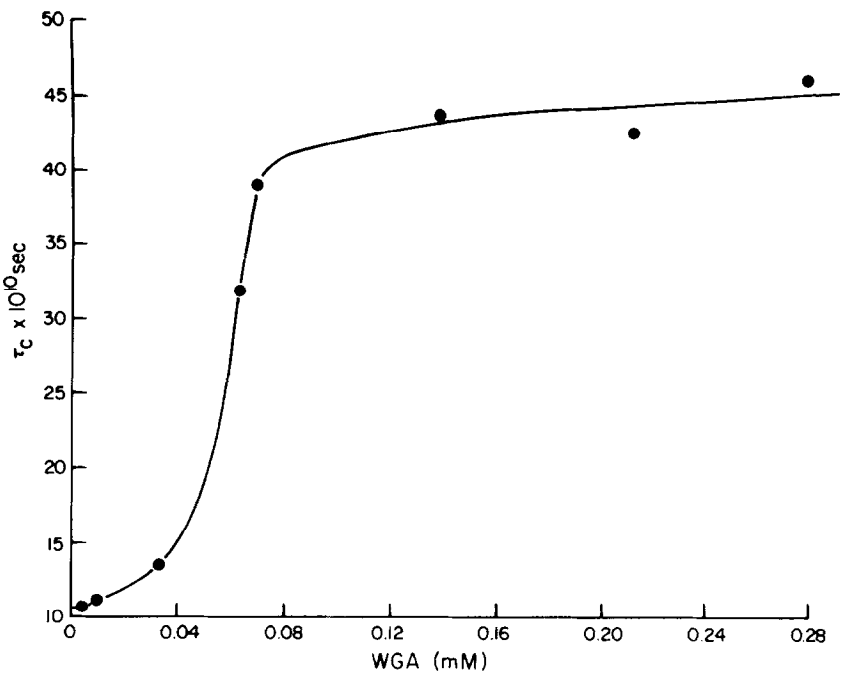

Figure 2 Oligosaccharide immobilization as a function of WGA concentration. Run at 21°C in HEPES buffered saline containing 2 mM Ca^{2+} and Mg^{2+}.

centration of WGA. As might be expected, the degree of terminal sugar immobilization is a function of the amount of lectin added - reaching a maximum at a point corresponding to total receptor site occupancy. The result is a sigmoidal curve, indicating that the process is positive cooperative. In this regard it may be worth remarking that positive cooperativity is a frequently reported feature of lectin binding to whole cells (21-24), and has been tentatively correlated with the mechanism of cellular response to specific contact events. Figure 2 indicates that the basic lectin/receptor interaction itself can be cooperative in nature, presumably as a result of a lectin induced conformational change such as headgroup clustering.

CONCLUSIONS

One is left with the impression that in the intact cell the headgroup of the transmembrane glycoprotein, glycophorin, occupies an extended and mobile configuration - one which permits its oligosaccharide chains considerable freedom of motion. At 37°C the correlation times for individual sugars may be expected to range from some 6×10^{-10} sec to 8×10^{-10} sec.

In explaining cellular effects of specific contact events it is common to suggest that binding to a receptor glycoprotein may induce a functional change in local membrane architecture (1,2). Spectroscopic studies of covalently labelled receptors should provide insight into the possibility of such a mechanism since the probe can be localized to the (small) region of interest. Our observations of strong oligosaccharide immobilization by WGA, and positive cooperativity in the binding event itself, support a mechanism which invokes direct lateral rearrangement of receptors. However our observation that oligosaccharide dynamics are totally divorced from factors affecting the membrane-inserted hydrophobic portion of the polypeptide backbone would argue against direct perturbation of the lipid annulus by a binding event.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Cancer Institute and Medical Research Council of Canada.

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