SELECTIVE SPIN LABELING OF SIALIC ACID RESIDUES OF GLYCOPROTEINS AND GLYCOLIPIDS IN ERYTHROCYTE MEMBRANES

A novel method to study cell surface interactions

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1. Introduction

Sialic acid, a negatively charged carbohydrate that occupies the terminal position on many of the polysaccharide chains attached to membrane glycoproteins and glycolipids, has been implicated in several membrane-associated processes including cellular fusion, contact inhibition, cation, hormone, neurotransmitter, and lectin binding, and immunochemical and tumorigenic properties ([1–7] and references therein).

We report and characterize here a method for selective labeling of sialic acid residues of glycoproteins and glycolipids in erythrocyte membranes with a nitroxide spin label.

2. Materials and methods

2.1. *Materials*

The spin label 2,2,6,6-tetramethyl-4-aminopiperdin-1-oxyl (Tempamine) was obtained from Aldrich. Mass spectral, NMR and IR analysis demonstrated that the spin label was pure.

2.2. *Biochemical procedures*

Blood was obtained into heparinized tubes by venipuncture from healthy volunteers. Intact cells and erythrocyte membranes (ghosts) were obtained as in [8]. Estimation of membrane protein content [9] and sialic acid levels [10], and SDS–polyacrylamide electrophoresis [11] were performed as described.

Exhausative pronase digestions of ghosts were carried out to quantitate the amount of ganglioside-bound spin label. Membranes were treated with 2 vol. of pronase (0.05 mg/ml) in 5 mM sodium phosphate, pH 8.0 (5P8) at room temperature for 1 h. The supernatant was carefully removed and the ghosts were resuspended in freshly prepared pronase for a second incubation under the same conditions. After again removing the supernatant the ghosts were washed once with 5P8 then diluted to their original volume. Additional pronase treatment released no electron spin resonance (ESR) signal into the supernatant. These ghosts were then made 1.0 N in NaOH and their ESR signal intensity (measured as the product of the square of the peak-to-peak linewidth and peak-to-peak amplitude of the low field line) determined. Signal intensities were compared to those of ghosts treated identically with omission of the enzyme. Lineshapes were the same in both cases. Gangliosides were separated from neutral lipids of spin labeled membranes as in [12].

2.3. *Spin labeling of sialic acid residues*

The C-7, C-8, and C-9 vicinal hydroxyl groups of sialic acid residues of isolated membranes (2.5–4.0 mg protein/ml) were oxidized to a terminal aldehyde by treatment with 1.0 mM $NaIO_4$ (final concentration) for 10 min at 0°C as in [13]. One volume of periodate-activated ghosts were spin labeled by reductive amination [14] overnight at 4°C with 2 vol. 1.0 mM tempamine and 2 vol. 1.0 mM $NaBH_3CN$ (both in 5P8), giving ~5-fold excess of each to sialic acid. The labeled membranes were then washed with 5P8 until no ESR signal could be detected in the supernatant. Use of larger concentrations of the

reducing agent $NaBH_3CN$ gave ESR spectra with higher signal/noise ratios. However, considerable Heisenberg spin exchange was noted under these conditions, suggesting additional spin probe binding sites per glycolipid or glycoprotein molecule were labeled when higher $NaBH_3CN$ concentrations were employed. All spectra used in these experiments gave minimal spin exchange compatable with signal detection. The degree of non-specific spin-label incorporation was determined by submitting ghosts not pre-activated by periodate to the tempamine/$NaBH_3CN$ incubation.

2.4. *ESR measurements*

ESR spectra were recorded on a Varian E-109 ESR spectrometer equipped with a Varian E-238 rectangular resonant cavity. Modulation and power broadening of spectral lines were avoided. The motion of the spin label was characterized by calculation of an 'apparent' rotational correlation time, τ_A:

$$\tau_A = 6.5 \times 10^{-10} W_o \left[\sqrt{\frac{h_o}{h_{-1}}} + \sqrt{\frac{h_o}{h_{+1}}} - 2\right]$$

where W_o, h_o, h_{+1}, and h_{-1} are, respectively, the peak-to-peak linewidth of the $M_I = 0$ line in Gauss and the peak-to-peak amplitude of the $M_I = 0$, +1, or −1 lines (see discussion in [15]).

2.5. *Characterization of the extent of spin labeling*

To 0.2 ml spin labeled ghosts and 0.1 ml 5P8 was added 20 μl NaB^3H_4 (4.8 mCi ^3H) and another 100 μl 5P8. After shaking at room temperature for 30 min, the ghosts were washed with 5P8 until only background radioactivity was observed in the supernatant. 100% incorporation of NaB^3H_4 was taken as that amount of radioactivity which resulted in ghosts from reaction of periodate-activated, non spin-labeled membranes with NaB^3H_4 [16].

Resulting SDS gels were immediately frozen [17] and sliced in 1 mm sections each of which was allowed to dry and then dissolved in 0.1 ml 30% H_2O_2 [18] at room temperature overnight. Biofluor (New England Nuclear) (4 ml) was added to each vial and after cooling the samples for several hours, the samples were counted on a Packard Model 3255 liquid scintillation counter.

3. Results and discussion

The ESR spectrum of ghosts labeled by the described procedure is shown in fig.1. Samples were diluted to ≤2.5 mg protein/ml to minimize spin exchange effects observed at higher membrane concentrations. The linewidth parameters essential to the analysis of these spectra are very dependent on ghost concentration; consequently, samples to be compared must always be adjusted so that protein contents are identical.

3.1. *Selectivity and extent of labeling*

Essentially no ESR signal is observed from ghosts subjected to tempamine/$NaBH_3CN$ incubation without prior activation by sodium periodate. This finding indicates that the non-specific incorporation of spin label into the membrane is negligible and suggests that, since sialic acid is the only constituent modified by periodate under the conditions employed [16,19,20], this sugar moiety is the site of labeling.

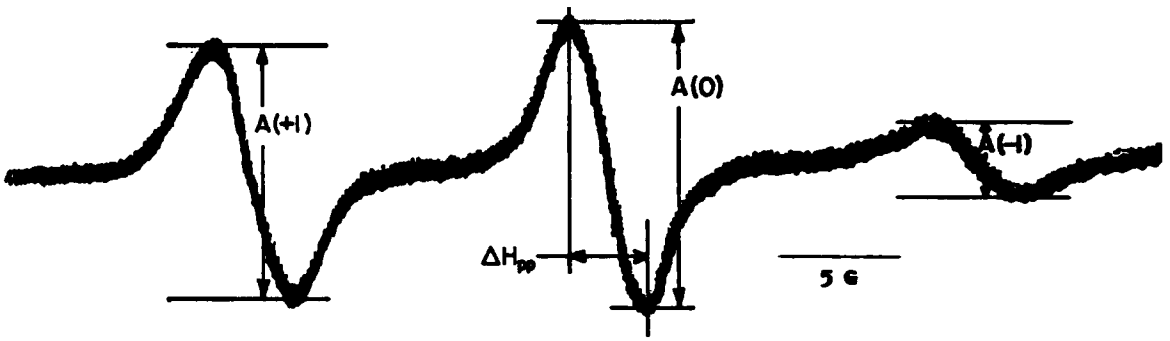

Fig.1. Typical ESR spectrum of tempamine spin label attached to sialic acid residues of membrane glycoproteins and glycolipids in human erythrocyte ghosts.

3.2. *Spin labeling of membrane sialoglycoproteins*

The distribution of tritium label introduced selectively into periodate-activated sialic acid residues of RBC membrane sialoglycoproteins by NaB^3H_4 reduction has been studied in [17,21]. We sought to characterize the distribution of tempamine among these proteins by studying the effect of prior spin-labeling on the tritium labeling pattern. The radioactivity profiles shown in fig.2 indicate that the spin label has blocked tritium incorporation into all sialoglycoproteins labeled by NaB^3H_4, and to a roughly proportionate degree. Thus all of the surface sialoglycoproteins appear to be labeled [17] with glycophorin, the most heavily sialated component of the RBC membrane, binding the majority of the spin label. In every case the amount of tritium incorporation being blocked was 32–44%, suggesting that ~40% of the sialic acid present was being spin labeled.

3.3. *Spin labeling of glycolipids*

Chloroform–methanol extraction of spin-labeled ghosts revealed that there was some lipid-bound label. Subsequent separation of glycolipids from the neutral lipids demonstrated that all of the ESR signal intensity resulted from the gangliosides. No non-specific labeling of phospholipids occurred. Analysis of the residual ESR signal following exhaustive pronase digestion of erythrocyte ghosts suggested that the spin labeled gangliosides account for ~30% of the total signal intensity.

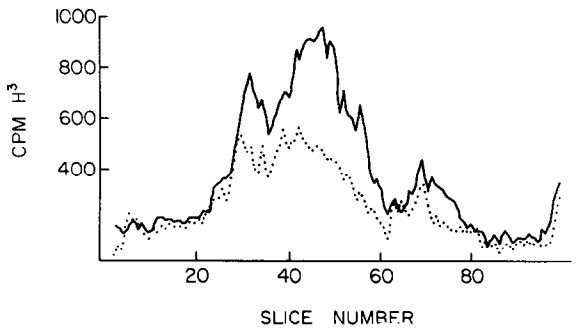

Fig.2. Illustrates the effect of prior spin labeling of sialic acid sites on the tritium incorporation profile of erythrocyte ghosts which has been subjected to $NaIO_4$ oxidation and NaB^3H_4 reduction followed by SDS–polyacrylamide gel electrophoresis as in section 2. (——) No prior spin labeling; (. . .) ghosts were spin labeled with tempamine as described prior to tritium incorporation.

3.4. *Analysis of ESR spectra*

Sialic acid residues are terminal sugar units of polysaccharides of glycoproteins and glycolipids and would be expected to be freely mobile. The most striking, and perhaps surprising, aspect of the spectrum observed from erythrocyte ghosts spin labeled as above (fig.1) is that the motion of the spin probe attached to sialic acid is hindered, presumably by the conformational properties of the membrane polysaccharide chains. The apparent correlation time of the rotational motion of tempamine bound to sialic acid in erythrocyte ghosts is ~0.84 ± 0.06 ns.

We have characterized our spectra of spin labeled ghosts in terms of an apparent (rather than actual) rotational correlation time (τ_A) since rigorous calculation of τ requires computer simulation of experimental spectra taking into account unresolved hyperfine couplings and solvent dependence of principle hyperfine values [22]. Although computational methods would undoubtedly enhance the accuracy of calculated correlation times, we feel that relevant biochemical information is obtainable from this system without such refinements.

The results of our experiments suggest that a specific, covalent labeling of sialic acid residues of erythrocyte membrane glycoproteins and glycolipids can be accomplished by sequential periodate oxidation and reductive amination. Other tissues might similarly be labeled and are being investigated in our laboratory. These procedures may help clarify cell-surface reactions such as lectin–membrane interactions, cell–cell fusion, and other membrane properties involving carbohydrate residues. Accordingly, we have begun a series of initial studies in this regard. The effect of *Phaseolus vulgaris* phytohemagglutinin (PHA) is to reduce the apparent correlation time of tempamine, by ~15%, suggesting increased motion of the sialic acid specific spin probe upon binding of the lectin.

While this study was in progress a paper describing but not characterizing the spin labeling of sialic acid residues of intact erythrocytes by periodate oxidation–reductive amination was published [23]. In addition to not characterizing the spin label sites, these authors use a much longer exposure to $NaIO_4$, conditions which activate galactose residues as well as sialic acid sites [20]. Upon repeating their procedures we observed marked Heisenberg spin exchange in contrast to our spectra. Dilution of samples prepared according to these authors yield spectra without sig-

nificant spin exchange effects. Such results indicate that caution must be used to avoid artifacts in ESR spectra obtained when employing these spin labeling methods.

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