# A GLYCOLIPID AND ITS ASSOCIATED PROTEINS: EVIDENCE BY CROSSLINKING OF HUMAN ERYTHROCYTE SURFACE COMPONENTS

C. A. LINGWOOD*, S. HAKOMORI and T. H. JI+

*Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA 98104* and *+Department of Biochemistry, University of Wyoming, Laramie, WY 82071, USA*

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## 1. Introduction

Glycosphingolipids function as antigens [1], receptors [2] and possibly regulators of cell proliferation and interaction [3]. They are chemically well defined, but they may represent only a part of the complex membrane machinery through which functions are achieved. Therefore, putative membrane proteins that may specifically interact with glycolipids have been postulated [4]. Their presence has been indicated by association of the specific membrane protein to a specific affinity glycolipid–glass column although the protein was not eluted nor isolated [5]. Two amphipathic low molecular weight (~4000) proteins have been isolated from ethanol and chloroform–methanol extracts of bovine erythrocytes that have some affinity to ganglioside (gangliophilin) and one of them displayed a strong Paul-Bunnell antigen activity [6]. The topology and organization of glycolipids and membrane proteins is now studied by heterobifunctional crosslinking reagents introduced in [7]. A large variety of crosslinking reagents have been used to study the arrangement and organization of proteins within cell membranes [8–10]. Methyl-4-azidobenzoimidate (MABI) terminates in a light-sensitive arylazido group which is coupled to an imidoester function which under alkaline conditions forms a linkage with any available amino group [7]. During ultraviolet irradiation the arylazido group is photolysed and will react with neighbouring C–H or C=C bonds [7,10] thus covalently linking adjacent components together. A crosslinking of peanut lectin and its receptor through a cleavable heterobifunctional reagent was reported in [11].

Here we report on the direct crosslinking of globoside or the indirect crosslinking through anti-globoside antibodies to the neighbouring globoside-associated protein (globophilic protein) to give a specific crosslinked complex.

*Abbreviations:* MABI, methyl-4-azidobenzoimide: PMSF, phenylmethylsulfonylfluoride; PBS, phosphate buffered saline (0.14 M NaCl, 2.6 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$ pH adjusted as indicated); RBC, red blood cells; globoside, a major glycolipid of human RBC membrane with a structure GalNAcβ1→3 Galα1→4Galβ1→4Glcβ1→1Cer [12]

* Present address: Biochemistry Department, Hospital for Sick Children, Toronto, 5MG 1X8, Ontario, Canada

## 2. Materials and methods

### 2.1. *Enzymatic treatment of RBC*

Fresh human RBC were washed by centrifugation 3 times with PBS (pH 7.4). Aliquots of packed cells (0.5 ml) were treated according to one of the following procedures: (a) resuspended in 1 ml PBS, pH 6.0 containing 25 units neuraminidase (Behring Diagnostics, Somerville, NJ) and 2 mM PMSF (Sigma, St Louis, MO); (b) resuspended in 10 ml PBS (pH 7.4) containing 0.25% trypsin (Difco, Detroit, MI); (c) resuspended in 1 ml PBS containing 2 mM PMSF as the control. All samples were incubated at 37°C for 30 min centrifuged and washed 3 times with PBS (pH 7.0).

### 2.2. *Surface labelling*

Galactose oxidase–$NaB^3H_4$, carried out in 1 ml PBS (pH 7.0), 20 units galactose oxidase (Kabi, Stockholm), 3 h at room temperature, followed by

resuspension in 1 ml PBS (pH 7.4) containing 1 mCi $NaB^3H_4$. After incubation at room temperature for 30 min unlabelled $NaBH_4$ was added and the cells were washed 3 times with PBS [13].

2.3. *Preparation of RBC ghosts*

This was by hypotonic lysis according to [14]. The ghosts were resuspended in 10 vol. 310 mosM phosphate buffer and allowed to reseal at 4°C for 30 min.

2.4. *Coupling of 4-methylazidobenzoimidate (MABI) and ultraviolet activation*

(a) Resealed RBC ghosts (from 0.5 ml packed cells) were resuspended in 1 ml PBS (pH 8.0) in the dark. MABI was dissolved in PBS (pH 8.0) (1 mg/ml). MABI solution (0.1 ml) was added to the RBC suspension at room temperature every 10 min for 1 h. The cells were incubated for a further 30 min and washed twice with PBS.

(b) Antigloboside (1 mg/ml) purified by affinity chromatography [15] was coupled with MABI as above and dialyzed extensively against PBS. The hemagglutination titre of the antibodies for trypsinized human RBC was checked before and after coupling and aliquots were frozen in a light-proof container. Resealed RBC ghosts were treated with MABI–antigloboside on ice overnight. MABI treated samples, either the procedure (a) or (b), were crosslinked as required by exposure to ultraviolet light (5 min, 1 cm from an Ultragrad II mineral light). RBC components were extracted in 1% Empigen [16] in 50 mM Tris (pH 8.0) at room temperature for 1 h (90–95% surface-labelled material released) or 4% SDS according to experimental design.

2.5. *Immunoprecipitation*

Globoside and proteins reactive to antigloboside were precipitated as in [17]. The Empigen RBC extracts were diluted and treated with the affinity-purified anti-globoside antibodies [15] or normal rabbit serum for 1 h at 4°C. Antigen–antibody complexes and excess antibodies were then selectively removed by addition of heat-killed *Staphylococcus aureus* bacteria [18]. The bacterial pellet was washed twice and bound material was eluted by boiling in 4% SDS. The eluted material was then separated by SDS slab gel electrophoresis in 8% polyacrylamide.

2.6. *Electrophoresis*

SDS–polyacrylamide electrophoresis was carried out essentially as in [19]. The slab gels were stained for protein with Coomassie blue and radiolabelled proteins detected on autoradiographs after fluorography [20].

## 3. Results and discussion

No evidence of crosslinking was seen in the detergent extract of surface-labelled RBC treated with MABI and ultraviolet light. Treatment with neuraminidase or trypsin prior to labelling and MABI addition did not facilitate crosslinking (fig.1). Only when membrane components which react with anti-globoside were selected could any change in electrophoresis pattern be detected after crosslinking. Precipitation of the Empigen released proteins with anti-globoside revealed a new labelled band from MABI treated cells

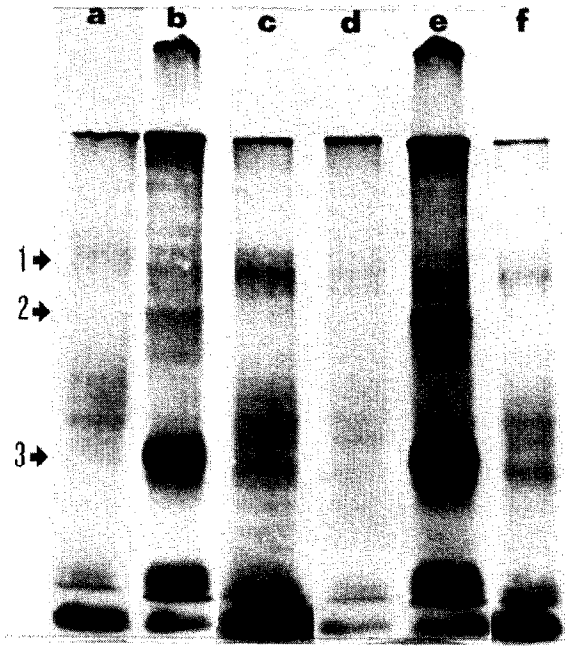


Fig.1. Polyacrylamide electrophoretic separation of RBC surface components. Arrows 1,2,3 indicate the position for band 3 protein, glycophorin A and B, respectively. RBC (untreated, or neuraminidase or trypsin digested) were labelled with galactose oxidase/$NaB[^3H]_4$ and treated with MABI with and without subsequent exposure to ultraviolet light as described in the text. Cells were extracted with 1% Empigen (1 h, room temperature) and samples applied to the gel. (a) Control; (b) neuraminidase-treated; (c) trypsin-treated; (d) control, ultraviolet irradiated; (e) neuraminidase-treated, ultraviolet irradiated; (f) trypsin-treated, ultraviolet irradiated.

after exposure to ultraviolet light. The new band migrates faster than other precipitated and labelled proteins and is of app. mol. wt 50 000. Neuraminidase treatment of RBC ghosts generally enhanced the glycoprotein labels which were reactive to anti-globoside. This is corresponding to the 'globoprotein' as in [18]. However, the intensity of the crosslinked component with app. mol. wt 50 000 was not much different, and no additional crosslinked component was observed after neuraminidase treatment (fig.2b). The crosslinking was still detectable for trypsinized RBC although the overall labelling was much reduced (fig.2d). The most reasonable explanation of these results is that

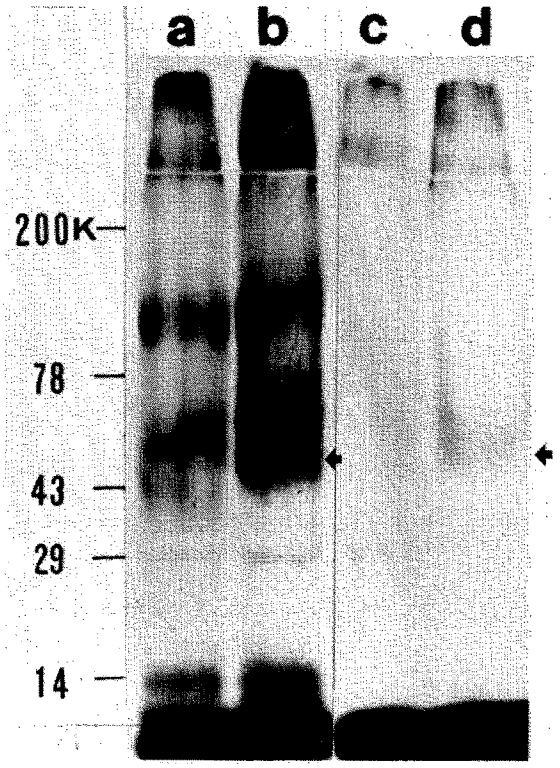


Fig.2. Effect of crosslinking with MABI on the SDS-electrophoretic pattern of RBC cell surface-labelled glycoproteins reactive to anti-globoside. RBC were treated with neuraminidase or trypsin prior to galactose oxidase labelling, treated with MABI with or without ultraviolet irradiation, extracted with 1% Empigen, and treated with anti-globoside or normal rabbit serum followed by precipitation with *Staphylococcus aureus* (see text). (a) neuraminidase-treated non-irradiated; (b) neuraminidase-treated and irradiated; (c) trypsinized non-irradiated; (d) trypsinized and ultraviolet irradiated. The position of molecular markers is indicated at the left margin. The arrows indicate the location of the crosslinked component.

globoside is directly coupled to a neighbouring low molecular wight protein or proteins to form a new higher molecular weight complex.

Attempts to crosslink proteins by treating surface-labelled RBC ghosts with anti-globoside pre-coupled to MABI proved successful only for trypsin treated cells when the exposure of globoside is maximal (fig.3). A new band, which is indicated by arrow in fig.3c,g, is evident after crosslinking corresponding to an app. mol. wt 76 000. If this complexed band is assumed to contain one immunoglobulin light chain linked to one surface protein complex, the RBC protein incorporated into the complex would also have mol. wt ~50 000 (76 000–25 000 = 51 000) which could be a similar complex as shown in fig.2. Low molecular weight amphipathic proteins, being soluble both in chloroform–methanol and in water, have been isolated from bovine erythrocyte membrane which displayed a binding affinity to glycolipid, particularly $GM_3$-ganglioside [6]. The crosslinked complex demonstrated in the precipitate with anti-globoside may contain globoside and globophilic protein, with mol. wt 50 000. Identification of crosslinked components through a cleavable crosslinking reagent remains to be studied. A possible role of glycolipid-associated protein in transmembrane control of cellular metabolism and in intercellular recognition through glycolipid is an important topic in future study.

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## References

[1] Marcus, D. M., and Schwarting, G. A. (1976) Adv. Immunol. 23, 203–240.
[2] Van Heyningen, W. E. (1974) Nature 249, 415–417.
[3] Hakomori, S. (1975) Biochim. Biophys. Acta 417, 55–89.
[4] Hakomori, S., Kijimoto, S., and Siddiqui, B. (1972) in: Membrane Research (Fox, C. F. ed) pp. 253–277, Academic Press, New York.

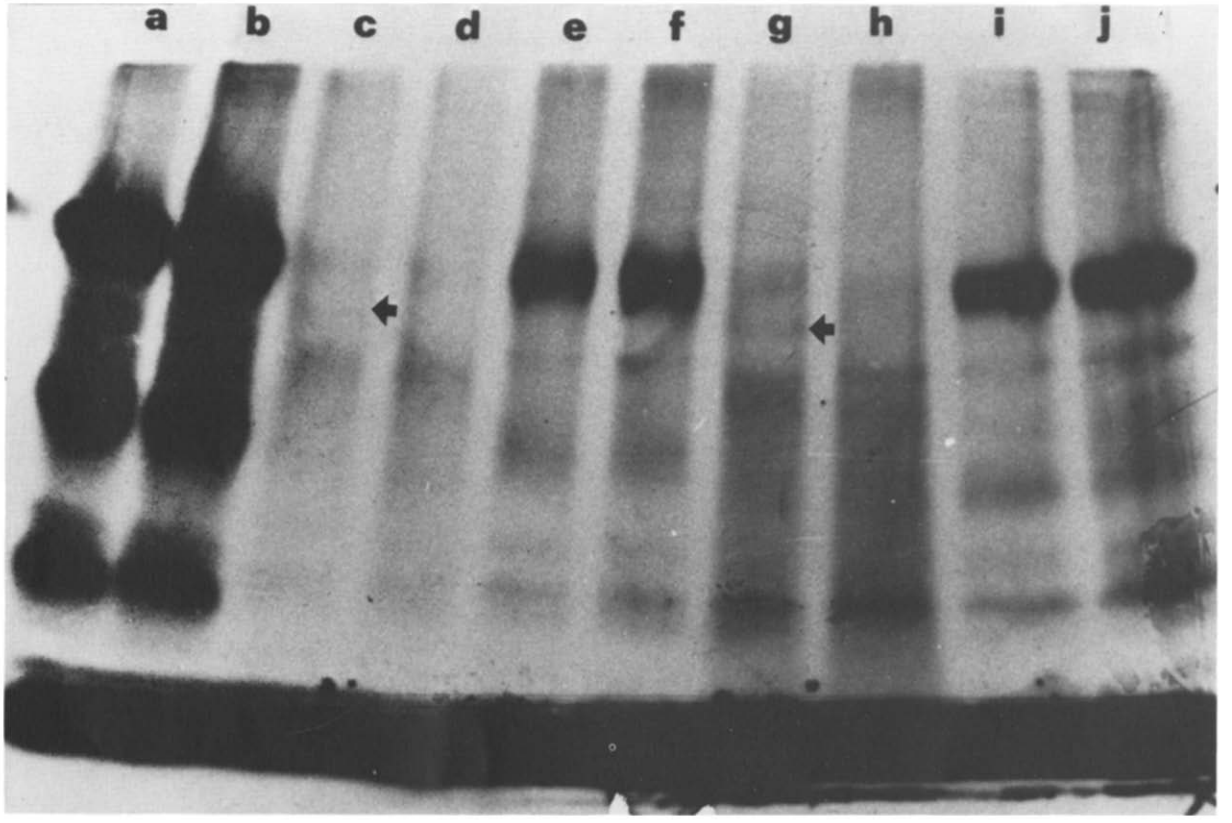


Fig.3. Crosslinking using anti-globoside coupled to MABI. Red blood cells were treated with neuraminidase or trypsin before or after surface labelling with galactose oxidase. After lysis the resealed ghosts were treated with MABI–anti-globoside (see section 2) with and without subsequent ultraviolet irradiation. Surface components were solubilized as before and separated by electrophoresis. (a) Neuraminidase-treated prior to surface labelling, ultraviolet-irradiated prior to extraction; (b) as (a) without ultraviolet irradiation; (c) trypsin-treated prior to surface labelling, ultraviolet-irradiated prior to extraction. (d) as (c) without ultraviolet irradiation; (e) neuraminidase-treated after surface labelling, ultraviolet-irradiated prior to extraction; (f) as (e) without ultraviolet irradiation; (g) trypsin-treated after surface labelling, ultraviolet-irradiated prior to extraction (h) as (g) without ultraviolet irradiation; (i) control, ultraviolet irradiated; (j) as (i) without ultraviolet irradiation. Arrows indicate the location of the crosslinked components.

[5] Carter, W. G., Fukuda, M., Lingwood, C., and Hakomori, S. (1978) Ann. NY Acad. Sci. 312, 160–177.
[6] Watanabe, K., Hakomori, S., Powell, M. E. and Yokota, M. (1980) Biochem. Biophys. Res. Commun. 92, 638–646.
[7] Ji, T. H. (1977) J. Biol. Chem. 252, 1566–1570.
[8] Ji, T. H. (1974) J. Biol. Chem. 249, 7841–7847.
[9] Wang, K. and Richards, F. M. (1975) J. Biol. Chem. 250, 6622–6626.
[10] Ji, T. H. (1979) Biochim. Biophys. Acta 559, 39–69.
[11] Jaffe, C. L., Lis, H. and Sharon, N. (1979) Biochem. Biophys. Res. Commun. 91, 402–409.
[12] Hakomori, S., Siddiqui, B., Li, Y-T., Li, S-C. and Hellerqvist, C. G. (1971) J. Biol. Chem. 246, 2271–2277.
[13] Gahmberg, C. G. and Hakomori, S. (1973) J. Biol. Chem. 248, 4311–4317.
[14] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119.
[15] Laine, R. A., Yogeeswaren, G. and Hakomori, S. (1974) J. Biol. Chem. 249, 4460–4466.
[16] Allen, J. C. and Humphries, C. (1975) FEBS Lett. 57, 158–162.
[17] Tonegawa, Y. and Hakomori, S. (1977) Biochem. Biophys. Res. Commun. 76, 9–17.
[18] Kessler, S. W. (1975) J. Immunol. 115, 1617–1624.
[19] Laemmli, U. K. (1970) Nature 227, 680–685.
[20] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.