CELL SURFACE LABELING OF ERYTHROCYTE GLYCOPROTEINS BY GALACTOSE OXIDASE AND Mn^{++}-CATALYZED COUPLING REACTION WITH METHIONINE SULFONE HYDRAZIDE*

Koichi Itaya, Carl G. Gahmberg† and Sen-itiroh Hakomori

Departments of Pathobiology and Microbiology,
University of Washington and Fred Hutchinson
Cancer Research Center,
Seattle, Washington 98195

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Summary - Methionine sulfone hydrazide (MSH) was coupled to 6-aldehydosugars and the reaction was found to be catalytically enhanced by Mn^{++} ion under physiological condition. The reaction was applied to label surface glycoproteins of erythrocytes with [^{35}S]-MSH after treating cells with galactose oxidase. The slab gel electrophoretic pattern of surface glycoproteins in sodium dodecylsulfate-polyacrylamide can be printed on autoradiogram. At least ten glycoproteins of normal human erythrocytes were printed; five (c, d, e, g, and k) were major bands, and of these four (c, d, e, and g) corresponded to "PAS I, II', II, and III". Others are hitherto unrecognized. Two intense bands each corresponds to c, and g, and two new bands, d' and e', were printed in desialylated fetal erythrocytes; intact fetal erythrocytes did not show significant label.

Increasing body of evidence indicates that control of growth behavior and proliferation of animal cells depends on the profile of surface glycoprotein and glycolipid (1-8). It is likely that the organization and assemblage of glycoprotein and glycolipid in membranes, rather than their primary structures, can be directly correlated with tumorigenicity and immunogenicity of animal cells. Thus, it is increasingly important to develop surface labeling method which can depict surface profile of animal cells with greater accuracy. A previous method, using galactose oxidase and tritiated borohydride (9, 10), has been successfully applied in studies of the surface profile of glycoprotein and glycolipid of various cells (1-3).

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†Present address: Department of Bacteriology and Serology, University of Helsinki, Finland. He was participating in this work while he was sponsored by International Forgaty Center Research Fellowship 1 F05 TW01885-02.

Abbreviations: MSH, methionine sulfone hydrazide; TLC, thin-layer chromatography; PAS, periodic acid sulfite reaction; PBS, phosphate buffered saline

A few drawbacks have been noticed, such as (1) for some cells non-specific label with tritiated borohydride alone is quite high, and is difficult to reduce[1]; (2) [^{3}H] label is not sufficient to obtain a good autoradiogram[2]. Consequently, coupling reagents for 6-aldehydogalactose or galactosamine have been sought with the following properties: 1) the reaction should occur under physiological pH (namely pH 6-8) and temperature (30-37°); 2) the reagent should not easily penetrate into cells; and 3) the reagent should be inexpensive and with isotope with strong energy emission, such as [^{35}S] or [^{125}I].

[^{35}S] Methionine sulfone hydrazide (4-methylsulfone-2-aminobutanoyl-hydrazide; see Fig. 1) was found to satisfy these requirements, but the extent of the coupling reaction was not appreciable for practical use. After a number of conditions were studied, we found that the coupling reaction can be greatly enhanced in the presence of $MnCl_2$ and sodium phosphate buffer (pH 6.0) at 37°. The reaction was successfully applied to label cell surface glycoprotein with [^{35}S] isotope, and enable us to print the labeling pattern by autoradiography. The surface glycoprotein profiles of normal human erythrocytes and that of fetal erythrocytes are presented.

MATERIAL AND METHODS

Synthesis of Methionine Sulfone Hydrazide: [^{35}S] Methionine from New England Nuclear (158 Ci/mM) was lyophilized in a small screw-capped glass tube, to the residue was added 50 µl of ice-cold performic acid solution. The performic acid solution was prepared by mixing 0.95 ml of concentrated formic acid (90%, Fisher Chemical Co.) and 50 µl of 30% hydrogen peroxide. The tube was kept in ice for 3 hours, whereby the sulfone was formed. After lyophilization, 150 µl of 1 N methanolic HCl was added and the tube was incubated at 80° for 2 hours. This treatment quantitatively converted the carboxyl group to the methyl ester. The preparation was evaporated under nitrogen and 150 µl anhydrous hydrazine was added (prepared from hydrazine hydrate and KOH by distillation under nitrogen stream). The mixture was kept at 80° for 3 hours

[1]Chemical structure of non-specific label with $NaB^{3}H4$ alone, is not known; a few possibilities have been discussed (1,9).

[2]Autoradiogram of [^{3}H]-labeled glycoprotein can be made by "fluorography" according to a recent procedure described by Bonner and Laskey (11). The gel should be immersed in dimethyl sulfoxide containing "PPO". Some glycoprotein with large amount of carbohydrate could be lost during the procedure. The method was however, successfully applied for erythrocyte glycoprotein (C.G. Gahmberg; unpublished observation), but is not warranted for labeling glycolipids of other animal cells.

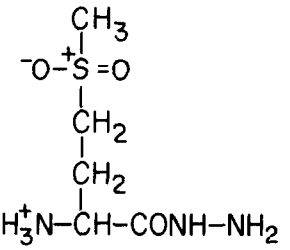

Figure 1.
Structure of methionine sulfone hydrazide (MSH)

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and was subsequently evaporated under nitrogen and stored in 1 ml of water at -20°. Cold methionine sulfone hydrazide was synthesized in a similar manner, starting with 200 mg of methionine and using 5 ml of performic acid, 2 ml of 1 N methanolic HCl and 0.5 ml of anhydrous hydrazine. All the synthetic steps were checked by thin-layer chromatography on Silica Gel G (ethanol-water 7:3). Rf value and the migration rate relative to methionine (in parenthesis) of various derivatives were as follows: methionine 0.59 (1.00), methionine sulfone 0.53 (0.90), methionine sulfone methylester 0.54 (0.92), methionine sulfone hydrazide 0.42 (0.71). The recovery of hydrazide was more than 90%.

Model Coupling Reaction With MSH and Erythrose or Methyl α or β-(6-aldehydo)-Galactoside Under Various Conditions With or Without Metal Ions: To a small screw-capped glass tube containing erythrose (50 µg) or methyl-(6-aldehydo)-galactoside (50 µg), 50 µl of sodium phosphate buffered solution (0.1 M, pH 6.0), 5 µl of MSH (50 or 100 µg) or [^{35}S]-MSH (10 µCi), and 5 µl of metal ion solution were added. The metal solution was added to a final concentration of 2 to 20 mM (see Fig. 2). The tubes were incubated at 37°. Five µl of the mixture was removed and applied to a TLC plate, Silica gel G. The TLC was developed with ethanol-water (7:3). The coupling product was characterized by atypical orcinol sulfuric acid reaction (brown red) and ninhydrin (orange), locating below 6-aldehydogalactoside or erythrose and above MSH.

Digestion With Trypsin: Half mg/ml of trypsin (Sigma) in PBS, pH 7.4 added to 0.2-0.5 ml of packed erythrocytes and the cells were incubated at 37° for 30 minutes. They were then washed 5 times in PBS pH 7.0 by centrifugation.

Treatment With Neuraminidase: Twenty-five units of Vibrio cholera neuraminidase (Calbiochem) in 0.5 ml of 0.1 M sodium phosphate buffer (pH 6.0) was added to intact cells. The cells were incubated at 37° for 30 minutes and were then washed 3 times in PBS pH 7.0.

Preparation of Cell Ghosts: The cells, labeled with [^{35}S]-MSH, were lysed in PBS (pH 7.4)-water 1:9 by vol. and centrifuged in a Sorvall centrifuge at 12,500 rpm for 20 minutes. The ghosts were washed 2 times in the same solution and kept at -70°.

Surface Labeling With [^{35}S]-MSH: Fifty to 100 µl of packed cells were suspended in 0.5 ml PBS, pH 7.0 and 10 Worthington units of galactose oxidase in 50 µl were added. The cells were incubated at 37° for 120 minutes, washed 3 times in PBS, pH 7.4, and suspended in 0.3-0.5 ml of sodium phosphate buffered solution (0.1 M, pH 6.0). $MnCl_2$ was added to a final concentration of 3-8 mM. Twenty µCi of [^{35}S]-MSH was added and incubated at 37° for 2-3 hours. Then this suspension was diluted with the same buffered solution and centrifuged to separate cells and ghosts were prepared as described above.

Slab Gel Electrophoresis: This was done according to Laemmli in 8% acryl

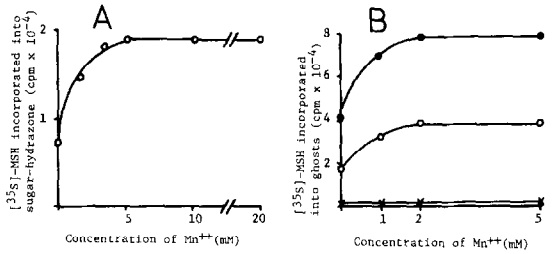


Figure 2.
Mn^{++}-catalyzed enhancement of the coupling reaction between MSH and aldehydosugars (A) and erythrocyte glycoproteins after galactose oxidase treatment (B). In (B), lines with solid circles, open circles and cross points indicate, respectively, erythrocytes treated with neuraminidase and galactose oxidase, those without neuraminidase but galactose oxidase alone, and those without galactose oxidase.

amide gels (12). To one volume of packed ghosts, was added the same volume of two times concentrated "final sample buffer solution" (12). The solution should be freshly prepared, and the pH carefully adjusted to between 6.7-6.8. The mixture was heated for 2 minutes in boiling water. Standard proteins labelled with [^{14}C] formaldehyde (13) were run in separate slots.

RESULTS

Manganese-catalyzed coupling reaction (hydrazone formation) of MSH and 6-aldehydo-galactoside and/or erythrose: The yield of the hydrazone separated on TLC, was greatly enhanced when the reaction was carried out in the presence of 5 mM $MnCl_2$, in sodium phosphate buffer (0.1 M, pH 6.0). The [^{35}S]-MSH radioactivity was detectable coincident with the hydrazone spot when [^{35}S]-MSH was used. As shown in Figure 2A, the radioactivity of the hydrazone spot was greatly enhanced when the reaction was carried out in the presence of $MnCl_2$ at pH 6.0. Neither the hydrazone spot, nor the corresponding radioactivity, was detected when the reaction was carried out in a simple physiological saline (0.14 M NaCl) or in a phosphate-buffered saline with various pH's between 6.5 and 7.4. Other metal salts such as 5 mM $FeCl_3$, $SnCl_2$, $CuSO_4$, and $ZnCl_2$, had no effect. Under the given conditions, no $MnPO_4$ precipitate formed.

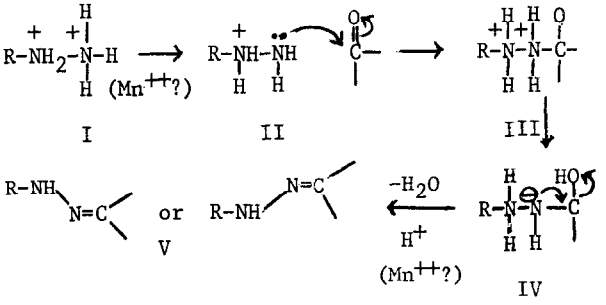
Manganese-catalyzed coupling of [^{35}S]-MSH to erythrocyte surface glyco-

proteins after oxidation with galactose oxidase: As seen in Figure 2B, the incorporation of [^{35}S] radioactivity from [^{35}S]-MSH into erythrocyte membranes was greatly enhanced by $MnCl_2$. The incorporation was extremely low without pretreatment of cells with galactose oxidase.

Autoradiogram of slab gel electrophoretogram of surface labeled glycoproteins by Mn^{++}-catalyzed coupling with [^{35}S]-MSH after galactose oxidase treatment: The autoradiogram of the slab gel electrophoretic pattern was successfully printed by autoradiogram when surface glycoprotein of erythrocytes was labeled by coupling with [^{35}S]-MSH after galactose oxidase treatment. A typical example is shown in Figure 3. At least ten glycoprotein bands can be labeled (designated a, b, c.....k). Bands c, d, e, g, and k were greatly intensified after sialidase treatment of cells. Intact fetal erythrocytes did not show any significant label, however, two intense bands corresponding to "band c and g" and two new bands (designated d', e') were printed after neuraminidase treatment.

DISCUSSION

Addition of amines, hydroxylamines or hydrazides to carbonyl group proceeds through a rapid attack of the carbonyl group by the nucleophilic $R-\overset{H}{\underset{H}{N}}:$ group followed by arrangement of conjugated acid $RN^{+}H_3$ and sluggish dehydration as shown below (in case of hydrazides):



The reactions above do not proceed satisfactorily without heating or without high concentration of H^{+}. We now found the reaction can be carried out at room temperature, under physiological conditions with Mn^{++} catalysis. A specific catalytic effect of manganese ion on this reaction, has not been

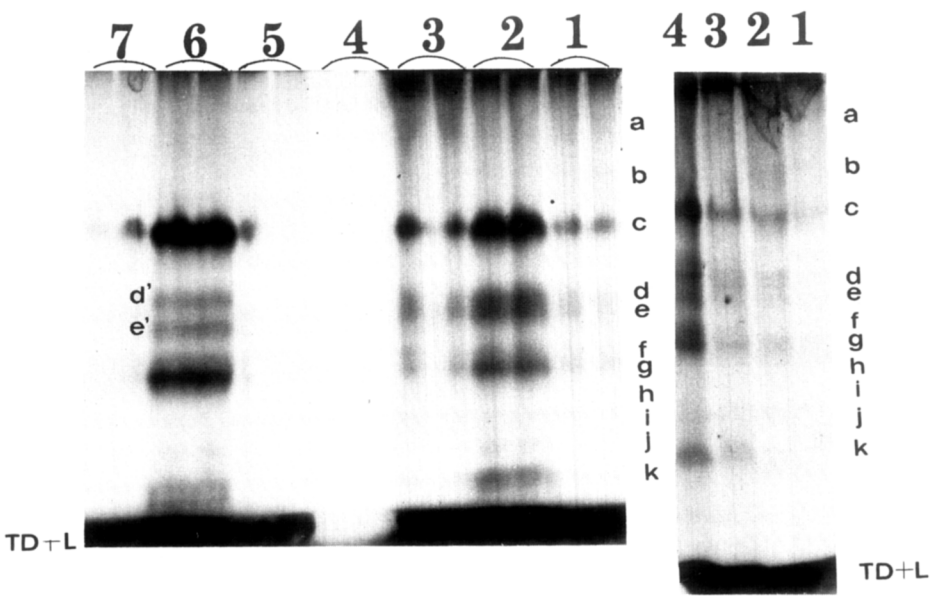


Figure 3.
Autoradiogram of slab gel electrophoresis pattern of erythrocytes glycoproteins.
Right: *Effect of Mn^{++} ion on labeling intensity with [^{35}S]-MSH after galactose oxidase.* 1. galactose oxidase and MSH alone, 2. galactose oxidase and MSH + 5 mM Mn^{++}. 3. neuraminidase treated, then galactose oxidase and MSH, 4. neuraminidase treated, then galactose oxidase and MSH + 5 mM Mn^{++}.
Left: *Comparison of adult and fetal erythrocytes.* 1. adult erythrocytes, 2. adult erythrocytes neuraminidase treated, 3. adult erythrocytes, trypsin treated, 4. blank control (no galactose oxidase, but MSH alone), 5. fetal erythrocytes, 6. fetal erythrocytes neuraminidase treated, 7. fetal erythrocytes trypsin treated. Faint bands such as "a, b, i, j" were not well reproduced in these prints.

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described previously and the mechanism of catalysis cannot be formulated at present. It may help to prevent decrease of nucleophilic $R\text{-}\ddot{N}H_2$ at acidic pH. The rate limiting step, IV to V, is catalyzed by H^+, which decreases the concentration of $R\ddot{N}H_2$ and increases non-reactive conjugated acid, $R\overset{+}{N}H_3$ (I). This conflicting situation could be counterbalanced by Mn^{++} ion in acidic phosphate buffer.

The reaction has been successfully applied to surface-labeling of glycoprotein with [^{35}S]-MSH to 6-aldehydogalactosyl residue of glycoprotein which

was created by galactose oxidase. The method has allowed us to print an autoradiogram on the slab gel since the labeling reagent contains [^{35}S]. Thus the method can be applied for exact comparison of surface glycoprotein profile.

The major label of human erythrocyte glycoprotein revealed by this method are the bands c, d, e, g, and k (Fig. 3,4), which correspond, respectively, to the "PAS 1, 4, 2, and 3 (or PAS I, II', II, and III) (14, and see for a review, 15). The faint bands a and b, correspond to the position of "protein 2-1" and "protein 3" (15) respectively, but await further identification. A strongly labeled glycoprotein k, and other minor bands f, h, i, j (see Fig. 4) were also detected by the present method, but they were not obviously PAS-positive. As the PAS reaction largely depends on sialyl residue, these glycoproteins may be less sialylated. A faint but distinct label occurred corresponding to "protein 3", is of particular interest. Although "protein 3" is a major protein of erythrocyte membrane and is

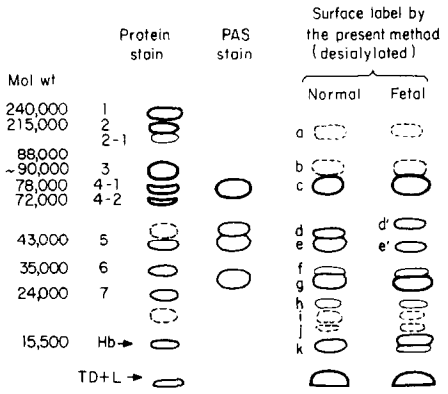

Figure 4.
Comparison of protein bands, PAS positive bands, and surface-labeled components by the present method after desialylated. For designations of protein bands and PAS bands see the review by Steck (15).
Intensity of bands expressed by the thickness of line; circles with bold line: very strong band, that with medium-thick line; bands with moderate intensity, that with thin line; bands with weak intensity, that with dotted line; faint bands. Hb: position of hemoglobin oligomer, TD + L: tracking dye and lipids.

difficult to stain by PAS reaction, it contains about 6-8% of carbohydrates (16). Recently, the protein was identified to carry majority of binding sites to Con A and Ricinus communis (17,18), whereas "PAS I", the major glycoprotein, carries relatively little binding sites to these lectins (19).

Intact fetal erythrocytes were not surface-labeled as previously shown (9), whereas intense bands, corresponding to bands c, g, and new bands d' and e' (see Fig. 3,4) were printed after fetal erythrocytes were treated with neuraminidase. These indicate that major surface glycoproteins of fetal erythrocytes are highly sialylated and some of them differ qualitatively from that of adult erythrocytes. These surface structures of fetal cells could be an important molecular basis of developing cells which characterizes some antigenic property of fetal cells (20).

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