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Selective Labeling of Functional Groups on Membrane Proteins or Glycoproteins using Reactive Biotin Derivatives and ^{125}I-Streptavidin[1]

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SUMMARY: Amino groups, sulfhydryl groups or oxidation-induced aldehydes on erythrocyte membrane proteins and/or glycoproteins, were reacted with biotinyl *N*-hydroxysuccinimide ester (BNHS), 3-(*N*-maleimido-propionyl) biocytin (MPB) or biocytin hydrazide (BCHZ), respectively. The detergent-lysed biotinylated samples were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were transferred onto nitrocellulose membranes. The blot was then incubated with a solution containing ^{125}I-streptavidin, and processed for autoradiography. The advantages of this approach over previously reported procedures for labeling the three functional groups include the following: (a) extremely high sensitivity; (b) short exposure times of autoradiograms and relatively low levels of radioactivity; (c) single-step radiolabeling procedures subsequent to processing and handling of gels and (d) no background labeling in control samples.

The study of the role of functional groups of membrane proteins has been hampered due to their relatively low levels which requires, in most cases, the use of very strong radioactive reagents. Many group-specific reagents have been used for this purpose. For example, the radioactive iodination of amino groups in proteins can be achieved by the use of the Bolton-Hunter reagent, an iodinated N-hydroxysuccinimide ester (1). In addition, sulfhydryl groups can be labeled with radioactive [^{14}C]-maleimides (2). Using another approach, oxidation-induced

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Abbreviations used: BCHZ, biocytin hydrazide; BNHS, biotinyl *N*-hydroxysuccinimide ester; MPB, 3-(*N*-maleimido-propionyl) biocytin; NAGO, neuraminidase and galactose oxidase; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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aldehydes on glycoproteins can be reduced with tritiated borohydride thereby acquiring a radioactive hydrogen (3). In all of these procedures the functional groups on proteins or glycoproteins are directly radiolabeled prior to any further manipulation, like gel electrophoresis and autoradiography. In one respect, the use of radioactive materials provides some advantage in that the label can be followed directly throughout all steps in the procedure. On the other hand, one is exposed constantly to high levels of radioactivity.

In this communication, we describe a new strategy for the electrophoretic detection of the three functional groups using the avidin-biotin complex as a mediator. In this approach, biotin-containing reagents are used throughout the procedure. The reagents are non-radioactive, such that the biotinylated macromolecules are not directly radiolabeled. This fact facilitates manipulation of the samples. Radioactive ^{125}I-streptavidin is used for detection of biotinylated material on the blots only at the last step. Biotin-modified erythrocyte membranes are employed as a model system to demonstrate the efficacy of the approach.

EXPERIMENTAL PROCEDURES

Materials. Biotinyl *N*-hydroxysuccinimide ester (BNHS) (4) and 3-(*N*-maleimido-propionyl) biocytin (MPB) (5) were prepared as described earlier. Sodium periodate and 2-mercaptoethanol were obtained from E. Merck (Darmstadt, FRG); sodium borohydride from Fluka AG (Buchs, Switzerland). *Vibrio comma* neuraminidase was from Behringwerke AF (Marburg, FRG). *Dactilium dendroides* galactose oxidase and *N*-ethyl maleimide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nitrocellulose paper (BA-85) was purchased form Schleicher and Schuell (Dassel, FRG). Streptavidin was purified from the spent culture broth of *Streptomyces avidinii* using a modified iminobiotin-containing affinity column (6). Streptavidin was iodinated by the chloramine-T method (7) to yield ^{125}I-labeled protein with a specific activity of 2.5 μCi/μg streptavidin.

Preparation of Biocytin Hydrazide. Biocytin methyl ester was prepared from biocytin (5,8) in methanolic solution by reation with thionyl chloride. TLC on silica gel plates gave one spot (R_F = 0.48 in butanol/acetic acid/water, 4:1:1) with no visible contaminants using ninhydrin or dimethyl aminocinnamaldehyde spray (9).

Biocytin methyl ester (100 mg) was dissolved in 2 ml methanol, and hydrazine hydrate (0.1 ml) was added. After 48 h at 25°C, the solvent was concentrated to dryness and dried further in a desiccator under reduced pressure in the presence of H_2SO_4 until no smell of hydrazine could be detected. The product was dissolved in water (1 ml) and trace amounts of hydrazine were removed on a column containing Porapak Type Q (Waters Associates, Inc.). Biocytin hydrazide was crystallized from hot ethanol (R_F = 0.11 on TLC using methanol as solvent). Elementary analysis (C,H,N,S) was in accordance with expected values.

Reaction of Biotin Derivatives with Erythrocyte Membranes. The schemes illustrating the reactions between the different reactive biotin derivatives and the respective functional groups on proteins are depicted in Fig. 1. Erythrocyte membranes were prepared from fresh human erythrocytes according to Fairbanks et

al. (10). Biotinylation of these membranes with BNHS or MPB was carried out as follows: membrane pellets (0.1 ml) were suspended in 2 ml phosphate buffered saline (PBS) containing either 10 or 50 μg/ml of BNHS or MPB. The samples were incubated for 30 min at room temperature and were then extensively washed with PBS. To selectively modify S-S bonds (5), a fresh membrane preparation was incubated in PBS containing 10 mg/ml *N*-ethyl maleimide for 30 min. After three washes with PBS the S-S bonds were reduced by 2% 2-mercaptoethanol. After an extensive wash, the membranes were reacted with MPB as described above.

In order to oxidize the membranes, sodium periodate or the enzymes neuraminidase plus galactose oxidase (NAGO) were used. For periodate oxidation, 0.1 ml erythrocyte membrane pellets were suspended in 2 ml ice cold PBS containing 1 mM sodium periodate. The suspension was kept on ice for 30 min. For NAGO-oxidation, membrane pellets were suspended in PBS containing neuraminidase (0.03 U/ml) and galactose oxidase (3 U/ml), and incubated at 37°C for 30 min. Control samples contained neither periodate nor NAGO. The oxidized membranes were washed three times with PBS, and samples were then incubated for 30 min at room temperature in a PBS solution containing 50 μg/ml BCHZ. The samples were washed twice with PBS, and half of each sample was further reduced for 30 min at 4°C with 2 mM sodium borohydride in PBS.

Gel Electrophoresis, Protein Blotting and Autoradiography. SDS-PAGE was performed by the method of Weber and Osborn (11) using the discontinuous buffer system of Laemmli (12) and a 10% separation gel. The proteins were blotted electrophoretically onto nitrocellulose paper according to Gershoni and colleagues (13,14). The nitrocellulose paper was quenched overnight with 2% bovine serum albumin in PBS and then incubated for 2 h in a similar solution to which 10^6 cpm/ml of ^{125}I-streptavidin had been added. The nitrocellulose sheet was washed extensively, incubated overnight with 0.1% Tween 20 in PBS, air-dried and exposed for 24 h to Kodak X-OMAT film at -70°C with an intensifying screen.

RESULTS AND DISCUSSION

Biocytin hydrazide was used in this study instead of biotin hydrazide, since BCHZ contains a hydrazide group as well as an amino group, the combination of which contributes to its particularly strong reactivity with aldehydes. Indeed, the Schiff-base formed in the interaction between BCHZ and oxidation-induced aldehydes

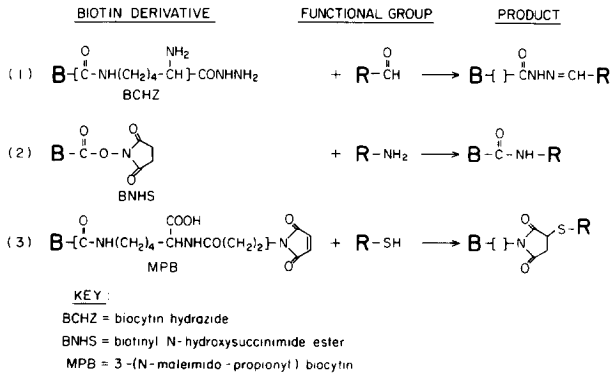

Fig. 1. Reactions of biotin derivatives with the respective functional groups on proteins and/or glycoproteins. B represents the biotin moiety without the carboxyl group, and R represents a cell surface macromolecule which possesses the designated functional group.

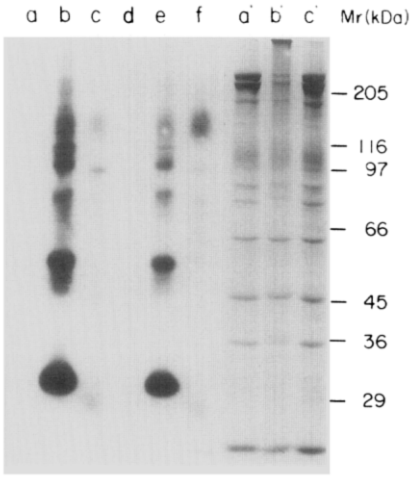

Fig. 2. Fluorography patterns of BCHZ-labeled erythrocyte membrane glycoproteins. Control and oxidized membranes were incubated with BCHZ with or without subsequent treatment with borohydride. The glycoproteins were separated by electrophoresis, blotted onto nitrocellulose paper, incubated with ^{125}I-streptavidin and processed for autoradiography. a, Non-oxidized membranes; b, periodate-oxidized membranes; c, NAGO-oxidized membranes; d-f, same as a-c but reduced subsequently with borohydride. a'-c', Coomassie brilliant blue-stained patterns corresponding to samples a-c.

(Fig. 1) needed no further stabilization by reduction with borohydride (Fig. 2). The extended side chain in BCHZ [relative to that of biotin hydrazide (15)] contributes to its improved detection properties when complexed with streptavidin, since steric constraints imparted by the deep biotin-binding pocket of the protein are thus relieved.

The data presented in Figure 2 also indicate the differences between periodate-induced and NAGO-induced oxidation of erythrocyte membrane glycoproteins. It is clear from the figure that periodate oxidizes more glycoproteins and with stronger intensity than those oxidized with NAGO. Similar observations were also obtained with human lymphocytes (data not shown). No background labeling was observed without oxidation.

To label amino and sulfhydryl groups, we used BNHS and MPB, respectively. Figure 3 demonstrates the erythrocyte membrane proteins labeled with these reagents; the molecules which contain disulfide bonds are shown in lane f.

In this communication we describe a convenient new method for the electrophoretic detection of different functional groups on membrane proteins and glycoproteins. The high sensitivity in the detection of the functional groups is

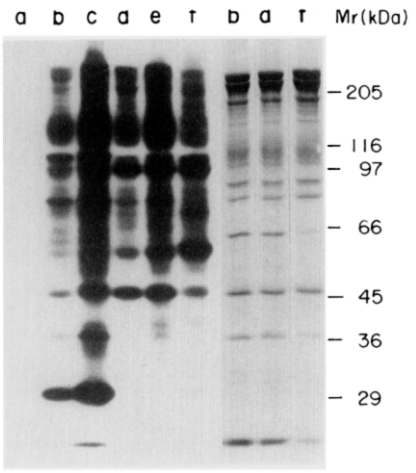

Fig. 3. Fluorography patterns of BNHS-labeled or MPB-labeled erythrocyte membrane proteins and glycoproteins. Membrane samples were subjected to SDS-PAGE and blotted as described in the legend to Figure 3. Membranes were either untreated (a), treated with BNHS at 10 μg/ml or 50 μg/mg (b and c, respectively), treated with MPB at 10 μg/mg or 50 μg/ml (d and e, respectively) or subjected to successive treatment with *N*-ethyl maleimide and 2-mercaptoethanol prior to incubation with MPB at 50 μg/ml (f). b', d' and f' show the Coomassie blue patterns of the corresponding autoradiograms.

exemplified in Figure 3. Incubation of erythrocyte membranes with BNHS or MPB at 10 μg/ml for 30 min was sufficient to induce maximal labeling. More importantly, the use of ^{125}I-streptavidin to detect the biotinylated macromolecules shortens the period of autoradiography to several hours, instead of a period of several weeks which is required in the case of a tritium label (16).

Streptavidin has several advantages over avidin as a probe. Streptavidin contains six tyrosines (17,18) per subunit and is amenable to extensive iodination (avidin has but one tyrosine which may be essential to its biotin-binding capacity). Unlike avidin, which is a basic glycoprotein, streptavidin is not charged and contains no sugar residues, yet its biotin-binding properties are remarkably similar to those of avidin. It thus avoids the potential problem of nonspecific attachment of the avidin molecule to anions.

^{125}I-Streptavidin has recently been used to detect biotin-containing carboxylases in plants (19). In addition, streptavidin has been conjugated to acid phosphatase and used to detect specific antigens on Western blots in conjunction with a biotinylated antibody (20). We have also used enzyme-conjugated avidin and streptavidin for protein blotting, thus omitting the use of radioactivity altogether

(unpublished results). Using this procedure, however, we sacrifice the extreme sensitivity of detection for proteins or glycoproteins which are present in minute amounts in biological materials. The fact that streptavidin can undergo such strong radioiodination enables the detection of trace quantities of biotin-containing compounds, the cost of which is only a very short exposure to relatively low levels of radioactivity. The ^{125}I-streptavidin molecule could be used for at least 3 months with highly reproducible results.

We have recently found in our laboratory that the procedure is suitable for biotin-containing DNA probes (data not shown). In previous work in this area, enzyme-based streptavidin- and avidin-containing probes were used to circumvent extensive manipulations with radioactive materials (21). The use of radioactive streptavidin in the last step of the procedure (after the hybridization with the biotinylated probe) enhances the signal tremendously. This approach is therefore particularly attractive for the study of all systems in which biotin can be incorporated.

REFERENCES

1. Bolton, A.E., and Hunter, W.M. (1973) Biochem. J. 133, 529-539.
2. Ando, Y., and Steiner, M. (1976) Biochim. Biophys. Acta 419, 51-62.
3. Gahmberg, C.G., and Andersson, L.C. (1977) J. Biol. Chem. 252, 5888-5894.
4. Bayer, E.A., and Wilchek, M. (1980) Methods Biochem. Anal. 26, 1-45.
5. Bayer, E.A., Zalis, M.G., and Wilchek, M. (1985) Anal. Biochem. 149, 529-536.
6. Hofmann, K., Wood, S.W., Brinton, C.C., Montibeller, J.A., and Finn, F.M. (1980) Proc. Natl. Acad. Sci. USA 77, 4666-4668.
7. Greenwood, F.C., Hunter, W.M., and Glover, J.S. (1963) Biochem. J. 89, 114-120.
8. Bayer, E.A., and Wilchek, M. (1974) Methods Enzymol. 34, 265-267.
9. McCormick, D.B., and Roth, J.A. (1970) Methods Enzymol. 18A, 383-385.
10. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
11. Weber, K., and Osborn, M.J. (1969) J. Biol. Chem. 224, 4406-4412.
12. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
13. Gershoni, J.M., and Palade, G.E. (1983) Anal. Biochem. 131, 1-15.
14. Gershoni, J.M., Davis, F.E., and Palade, G.E. (1985) Anal. Biochem. 144, 32-40.
15. Wilchek, M., and Bayer, E.A. (1986) Methods Enzymol. (in press).
16. Favero, J.J., Gordon, I.L., Parker, J.W., and O'Brien, R.L. (1982) Cell. Immunol. 68, 38-52.
17. Chaiet, L., and Wolf, F.J. (1964) Arch. Biochem. Biophys. 106, 1-5.
18. Green, N.M. (1975). Adv. Protein Chem. 29, 85-133.
19. Nikolau, B.J., Wurtele, E.S., and Stumpf, P.K. (1985) Anal. Biochem. us 149, 448-453.
20. Brower, M.S., Brakel, C.L., and Garry, K. (1985) Anal. Biochem. 147, 382-386.
21. Leary, J.J., Brigati, D.J., and Ward, D.C. (1983) Proc. Natl. Acad. Sci. USA 80, 4045-4049.