Vol. 91, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1979 Pages 402-409

IDENTIFICATION OF PEANUT AGGLUTININ RECEPTORS ON HUMAN ERYTHROCYTE GHOSTS BY AFFINITY CROSSLINKING USING A NEW CLEAVABLE HETEROBIFUNCTIONAL REAGENT

Charles L. Jaffe, Halina Lis and Nathan Sharon

Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel

Received September 14, 1979

SUMMARY: Peanut agglutinin was acylated with a new heterobifunctional, cleavable photosensitive crosslinking reagent, *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*'-oxysuccinimide ester. The lectin derivative binds specifically and reversibly to neuraminidase-treated human erythrocyte ghosts and upon irradiation covalent attachment of over 35% of the bound lectin occurs. The affinity-crosslinked ghosts were solublized in deoxycholate, immunoprecipitated with anti-peanut agglutinin antiserum, and analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. Bands containing both peanut agglutinin and membrane glycoproteins were detected with apparent molecular weights of 58 000, 85 000, 110 000 and 135 000. Upon subsequent cleavage with sodium dithionite, asialoglycophorin A (apparent M.W. 41 000 and 85 000) and a second glycoprotein (apparent M.W. 58 000 - 61 000) were tentatively identified as the receptors for peanut agglutinin in the intact membrane.

INTRODUCTION

Current approaches to the isolation and characterization of membrane receptors for effectors such as hormones, toxins, antibodies and lectins, require the initial disruption of the membrane. However, disruption may reveal cryptic or hidden sites containing "receptors" which are inaccessible to the effector on the intact cell. In addition, these procedures do not differentiate between high affinity, biologically-relevant receptors that function at low effector concentration and between low affinity receptors which may not be involved in the elicitation of biological responses. Also, all information regarding the molecular environment of the receptor and the dynamic properties of the membrane is lost in the course of isolation.

A promising approach to the study of receptors and their molecular environment in the membrane, is by affinity-directed crosslinking (1). To examine

Abbreviations used: PNA, peanut agglutinin; M-PNA, peanut agglutinin acylated with the crosslinking reagent *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N*'-oxysuccinimide ester; PBS, 5 mM phosphate buffered saline, pH 7.2; BSA, bovine serum albumin.

0006-291X/79/220402-08$01.00/0
Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

the feasibility of this approach, we have synthesized a new type of cleavable, heterobifunctional and photosensitive reagent, *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester (I) (Fig.1) and have attached I via its activated ester group to lectins. Here we report on the covalent binding of such a derivative of peanut agglutinin (M-PNA) to neuraminidase-treated human erythrocyte ghosts. The lectin-receptor complexes were isolated by immunoprecipitation with antibodies against PNA and partially characterized both before and after cleavage of the complexes.

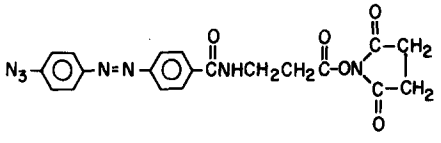

Figure 1 Structure of *N*-[4-(*p*-azidophenylazo)benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester.

MATERIALS AND METHODS

PNA was prepared by affinity chromatography on a column of Sepharose-*N*-(ε-aminocaproyl)β-D-galactopyranosylamine (2). The same support was used to purify M-PNA. [^{125}I]PNA was prepared by iodination with chloramine-T (3). The crosslinking reagent I, ε_{360} = 17 000, was synthesized (Jaffe *et al.*, in preparation) from 4'-(aminoazobenzene)-4-carboxylic acid (4). The experimentally determined chemical properties of I were in complete agreement with those predicted. All spectra were taken on a Cary 219 Ultraviolet/visible Spectrophotometer. Antibodies were prepared against PNA using a procedure essentially as described for soybean agglutinin (5). Two hundred µl of anti-PNA antiserum precipitated 50 µg of PNA.

PNA Derivative. Reagent I was coupled to PNA in the dark by the addition every 15 min of five aliquots (5 µl each) of 0.1 M I in *N*,*N'*-dimethylformamide to 12 mg PNA dissolved in 1.5 ml saline containing a specific inhibitor for the lectin (0.2 M lactose) and adjusted to pH 8.5 with 0.1 M $NaHCO_3$. After 1.5 hr at 4°C, the solution was centrifuged, applied to a Sephadex G-25 column and eluted with 5 mM phosphate buffered saline, pH 7.2 (PBS). Fractions absorbing at both 280 and 360 nm were pooled and the product further purified by affinity chromatography. M-[^{125}I]PNA was prepared by an identical procedure. The lectin derivatives migrated as single homogeneous bands on polyacrylamide gel electrophoresis and had the same hemagglutinating activity as the native, unmodified lectin when tested on rabbit erythrocytes (6).

Erythrocyte ghosts. Neuraminidase-treated human erythrocytes (outdated, type AB) were prepared by incubating 50 µl of "test-neuraminidase" (Behringwerke, AG) with 4 ml packed cells in two volumes of 0.1 M phosphate buffer, pH 6.0, for 30 min at 37°C. One half of the cells were labelled with tritium using the galactose oxidase (Kabi)-NaB[$^{3}H_4$] method (7) and resealed white ghosts were prepared (8) from both the labelled and unlabelled membranes.

Crosslinking to ghosts. M-[^{125}I]PNA (100 µg) was incubated in the dark at room temperature with neuraminidase-treated human erythrocyte ghosts (30 µl packed ghosts) in 200 µl of PBS containing 0.25% bovine serum albumin (PBS/BSA). After one hour, the agglutinated ghosts were washed by centrifugation (8 min at 1 200 x *g*) 2-3 times with PBS/BSA and irradiated for 1.5 min with a super pressure mercury lamp (200 W Osram) using the 365-366 nm band. The ghosts were resuspended in 1 ml PBS/BSA containing 0.4 M lactose and incubated at room temperature for 2 hr in order to remove all the noncovalently bound M-PNA. They were then washed by centrifugation (30 min, 30 000 x *g*) with 2 x 1 ml 0.2 M lactose in PBS/BSA followed by 1 x 1 ml PBS. Crosslinking of the M-PNA to neuraminidase-treated tritiated human erythrocyte ghosts was performed by an identical procedure.

Immunoprecipitation. Ghosts were solubilized in 200 µl of 0.5% sodium deoxycholate containing 0.4 M lactose and 1 mM phenylmethylsulfonylfluoride for 25 min at 37°C. The insoluble material was removed by centrifugation (30 min, 39 000 x *g*); the supernatant was collected and further centrifuged for 1 hr at 100 000 x *g* in a Beckman L2-65B Ultracentrifuge using a Ti60 rotor. The solubilized membrane fraction was incubated with 50-60 µl of either anti-PNA or normal rabbit serum for 1 hr at 37°C and then overnight at 4°C. The precipitates were collected by centrifugation at 8 700 x *g* for 4 min in a Beckman Microfuge, washed twice with 0.5% sodium deoxycholate containing 0.2 M lactose and 1 mM phenylmethylsulfonylfluoride, and analyzed by polyacrylamide gel electrophoresis. As an additional control, native PNA (15 µg) was added to uncrosslinked solubilized ghosts in the presence of lactose and precipitated by anti-PNA antiserum under conditions identical to those described above.

Cleavage. The anti-PNA lectin-receptor immunoprecipitate was dissolved in 100 µl of 2% sodium dodecylsulfate containing dithionite (100 mM) and allowed to stand at room temperature for 20 min or heated at 80°C for 5 min. After dialysis against doubly-distilled water (2 x 3 liters, 48 hr) and lyophilization, the material was dissolved in sample buffer for electrophoresis.

Sodium dodecylsulfate-polyacrylamide slab gel electrophoresis was performed using the system described by Fairbanks (9). Autoradiography of the M-[^{125}I]PNA gel and fluorography (10) of the tritiated ghost gels were performed by exposing preflashed Agfa-Gavaert Curix RPI X-ray film to the dried gels.

RESULTS

The spectrum of the M-PNA is given in Fig.2. Based on the ε_{max} at 360 nm of the crosslinking reagent I and the molecular weight of PNA (110 000, ref.2), different preparations of M-PNA and M-[^{125}I]PNA were shown to contain 3-4 moles of reagent I per mole of lectin, or one mole reagent per subunit of lectin. Irradiation of M-PNA, 0.67 mg/ml in PBS for 30 sec, results in a 50% reduction of the peak at 355 nm and a shift in the maximum to 340 nm. Photolysis of unmodified PNA for 5 min causes no change in its ultraviolet-visible spectrum (Fig.2).

The covalent binding of M-[^{125}I]PNA to the neuraminidase-treated erythrocyte ghosts was determined after 1.5 min irradiation (data not shown). Under

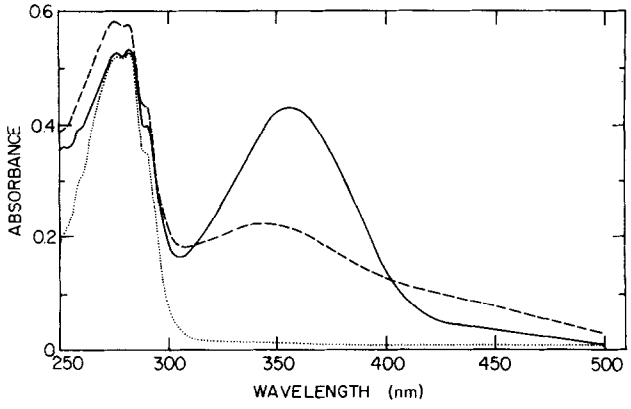

Figure 2 Absorbance spectra of M-PNA and PNA before and after irradiation. M-PNA (——); M-PNA following irradiation for 30 sec (---); native PNA before and after irradiation for 5 min (...). The proteins were dissolved in PBS (0.67 mg/ml) and irradiated with a super pressure Hg lamp (200 W Osram) using the 365-366 band.

these conditions the amount of M-[^{125}I]PNA covalently bound was 0.66 x 10^6 molecules M-[^{125}I]PNA/cell. When irradiation was carried out in the presence of 0.2 M lactose, 0.03 x 10^6 molecules of M-[^{125}I]PNA were bound per cell, indicating very low nonspecific binding. Prior to irradiation, 1.8 x 10^6 molecules M-[^{125}I]PNA/cell were bound to the neuraminidase-treated ghosts.

The Coomassie Blue stained gels of whole ghosts treated with M-[^{125}I]PNA are shown in Fig.3A; identical patterns were obtained with M-PNA. The appearance of a new band, migrating at a molecular weight identical to the PNA subunit (27 000), is clearly evident in the irradiated sample. This band is not seen in the absence of irradiation nor when irradiation is carried out in the presence of an inhibitory sugar. The Coomassie Blue patterns of the membrane fraction obtained by immunoprecipitation are also given in Fig.3A. Two intense bands are observed: the faster moving band represents a mixture of the PNA subunit and the light chain of the antibody (27 000 and 25 000 D respectively), while the slower band corresponds to the antibody heavy chain (50 000 D). No Coomassie Blue staining bands are observed when normal serum is used.

Autoradiography of the gels of M-[^{125}I]PNA-treated whole ghosts (Fig.3B) reveals in the irradiated samples the presence of new bands with apparent molecular weights of 52 000, 61 000, 79 000, 85 000, 110 000 and 135 000, in

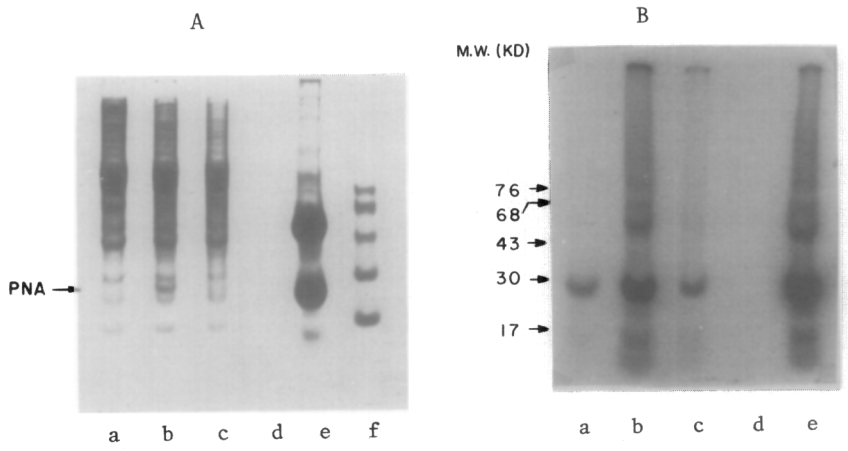

Figure 3 Sodium dodecylsulfate electrophoresis of neuraminidase-treated, human erythrocyte ghost preparations incubated with M-[^{125}I]PNA. (A) Coomassie Blue stained gels: (a) whole ghost preparations, no irradiation; (b) whole ghost preparations, irradiated for 1.5 min; (c) whole ghost preparations, irradiated for 1.5 min in the presence of 0.2 M lactose; (d) immunoprecipitate with normal serum; (e) immunoprecipitate with anti-PNA serum; (f) molecular weight standards: myoglobin 17 000 D, soybean agglutinin 30 000 D, ovalbumin 43 000 D, BSA 68 000 D, transferrin 76 000 D. The experimental conditions are given under Materials and Methods. (B) Autoradiography of the gels described above.

addition to a strong band due to the PNA monomer (27 000 D). Irradiation in the presence of lactose gives only a faint band migrating at 27 000 D. A similar faint band is also present in the non-irradiated samples. Autoradiography of sodium dodecylsulfate-polyacrylamide gels of the immunoprecipitates, containing approximately three times the amount of [^{125}I]PNA applied to the above gels of whole crosslinked ghosts, shows essentially the same pattern as that obtained without immunoprecipitation.

When neuraminidase-treated tritium labelled erythrocyte ghosts are used, fluorography of gels of the immunoprecipitated M-PNA–receptor complexes (Fig. 4) reveals five strong bands at 40 000, 58 000, 85 000, 110 000 and 135 000 D. In control experiments in which unmodified PNA, in an amount (15 µg) equal to that of the M-PNA crosslinked to the ghosts above, is mixed with solubilized neuraminidase-treated tritiated ghosts in the presence of lactose and precipitated with anti-PNA antiserum, no radioactive bands are visible upon gel electrophoresis of the immunoprecipitate. Cleavage of the immunoprecipitated

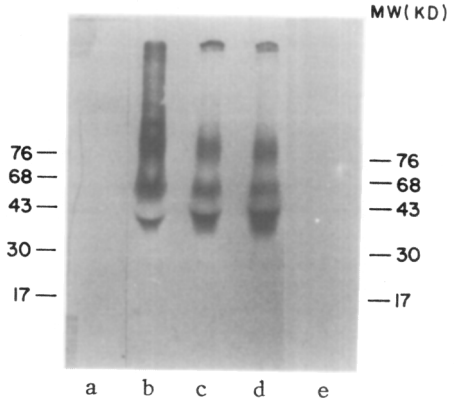

Figure 4 Fluorograms of sodium dodecylsulfate gels of neuraminidase-treated, tritium-labelled human erythrocyte ghosts crosslinked with M-PNA: (a) immunoprecipitate with normal serum; (b) immunoprecipitate with anti-PNA antiserum; (c) immunoprecipitate with anti-PNA antiserum followed by cleavage for 20 min at room temperature with 100 mM sodium dithionite; (d) immunoprecipitate with anti-PNA antiserum followed by cleavage for 5 min at 80°C with 100 mM sodium dithionite; (e) control with native PNA (15 µg) added to the uncrosslinked solublized ghosts in the presence of 0.2 M lactose and precipitated with anti-PNA antiserum without irradiation.

M-PNA–receptor complexes with sodium dithionite (Fig.4) results in the disappearance of the 110 000 and 135 000 D bands, along with most of the band at 85 000 D, and an increase in intensity of the 40 000 D band. The 58 000 D band appears to remain unchanged.

DISCUSSION

The results presented in this communication clearly demonstrate the covalent crosslinking of a photosensitive derivative of PNA to glycoproteins in ghosts obtained from neuraminidase-treated human erythrocytes. The best evidence for crosslinking is the appearance of high molecular weight radioactive bands (Fig.4b) in preparations obtained by immunoprecipitation following the irradiation of M-PNA bound to ghosts in which the surface carbohydrates had been radioactively labelled according to the method of Gahmberg and Hakomori (7). Some of the bands (apparent molecular weights 58 000 - 61 000, 85 000, 110 000 and 135 000) also contain the lectin, as can be seen from the analysis of unlabelled ghosts crosslinked with M-[^{125}I]PNA. Other [^{125}I]-labelled bands (52 000 and 79 000 D) probably represent crosslinked oligomers

of the PNA subunit. Since immunoprecipitation is carried out in the presence of lactose, a specific sugar inhibitor of PNA, only glycoproteins crosslinked to M-PNA are isolated.

Tentative identification of the molecular size of the membrane components to which the PNA is crosslinked is made possible by the fact that the crosslinking reagent can be readily cleaved by dithionite. After cleavage two tritium labelled bands, with apparent molecular weights of 85 000 and 40 000, are found, which compare favourably with the 82 000 and 46 000 D bands isolated by affinity chromatography of solubilized neuraminidase-treated ghosts on PNA-Sepharose (11). These two bands may correspond to the asialoglycophorin A monomer and dimer (12). A third band, with an apparent molecular weight of 27 000, which was isolated by affinity chromatography, is not obtained by affinity-directed crosslinking. This band may not be a receptor for PNA in the intact membrane but rather an artifact due to the disruption of the erythrocyte membrane prior to chromatography. Since no change is observed in the 58 000 D band following cleavage, it is also not certain whether this material is a receptor for PNA or a glycoprotein strongly associated with the receptor in the intact cell membrane. It is also possible that this band is due to the interconversion of the glycophorin A monomer and dimer during electrophoresis (12).

The tritium-labelled band, with an apparent molecular weight of 40 000 prior to cleavage, is probably non-crosslinked material, since cleavage does not afford bands with lower molecular weights; moreover, no band of similar molecular weight is seen in gels with [^{125}I]PNA. This non-crosslinked material may be an asialoglycophorin A monomer derived from immunoprecipitation of the asialoglycophorin A dimer in which only one of the monomers is crosslinked to M-PNA.

A previous attempt to identify the receptor for a lectin (concanavalin A) on the surface of human red blood cells by affinity crosslinking did not give conclusive results (13). Although a new band was observed by polyacrylamide

gel electrophoresis following irradiation of the lectin–ghost complex, it was not ascertained whether the band consisted solely of crosslinked lectin or of a lectin–receptor complex. Moreover, the reagent used was not readily cleavable.

Other heterobifunctional cleavable crosslinking reagents have been described in the past, but all of them contain disulfide bridges (1), which precludes the application of disulfide reducing reagents during the isolation and identification of the complexes; in addition these reagents are susceptible to disulfide exchange. The novel cleavable reagent described in this communication does not have any of the above disadvantages.

Besides its application in the detection of cell surface receptors for various effectors, affinity-directed crosslinking can also be used, by varying the length of the crosslinking agent, for examination of the molecular environment of cell membrane receptors *in situ*. Finally, since photo-crosslinking is short-lived and the moment of activation can be controlled, affinity crosslinking can also be used to study changes in receptor population and environment during dynamic membrane processes such as malignant transformation, lymphocyte stimulation and cellular development.

REFERENCES

1. Ji, T.H. (1979) Biochim. Biophys. Acta 559, 39-69.
2. Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518-8523.
3. Reisner, Y., Lis, H. and Sharon, N. (1976) Exp. Cell Res. 97, 445-448.
4. Thiel, V.A. and Peter, O. (1928) Z. Anorg. Ch. 173, 169-198.
5. Nachbar, M.S. and Oppenheim, J.D. (1973) Biochim. Biophys. Acta 320, 494-502.
6. Sever, J.L. (1962) J. Immunol. 88, 320-329.
7. Gahmberg, C.G. and Hakomori, S.I. (1973) J. Biol. Chem. 248, 4311-4317.
8. Sze, H. and Solomon, A.K. (1979) Biochim. Biophys. Acta 550, 393-406.
9. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
10. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
11. Carter, W.G. and Sharon, N. (1977) Arch. Biochem. Biophys. 180, 570-582.
12. Silverberg, M. and Marchesi, V.T. (1978) J. Biol. Chem. 253, 95-98.
13. Ji, T.H. (1977) J. Biol. Chem. 252, 1566-1570.