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# IDENTIFICATION OF MEMBRANE PROTEINS OF HUMAN BLOOD PLATELETS WITH A HYDROPHOBIC PHOTOLABEL

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**A photoactivable glycolipid probe, 12-(4-azido-2-nitrophenoxy)stearoyl[1-$^{14}$C]glucosamine, was used to label proteins and lipids of platelet membranes. The proteins were analyzed by two-dimensional high-resolution gelelectrophoresis. The labeling patterns showed that three membrane proteins were labeled which were not previously identified by ectolabeling (Sixma, J.J. and Schiphorst, M.E. (1980) Biochim. Biophys. Acta 603, 70–83). Analysis of the lipid fraction showed that phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were labeled by the probe. The distinct labeling of phosphatidylserine strongly suggests that the probe redistributes between the two halves of the bilayer.**

## Introduction

Membrane glycoproteins are essential for principal functions of blood platelets such as adhesion and aggregation [1–4]. The use of a combination of radioactive ectolabeling techniques with two-dimensional high-resolution gelelectrophoresis has recently allowed the identification of some 40 surface glycoproteins [5,6]. Such studies, however, do not provide information about membrane proteins that are not exposed on the surface. In a previous paper we approached this problem by combining information obtained by isolation of membranes on polylysine beads and by selective solubilization [7]. Several cytoplasmic proteins, among which α-actinin, were found to behave as peripheral proteins. Three minor integral proteins were recognized that were not exposed on the surface.

In this paper we studied membrane proteins with the use of a hydrophobic photoactivable glycolipid probe (Fig. 1). This photolabel is supposed to be specific for the outer leaflet of the membrane bilayer [8–10]. With this label we were able to identify three membrane proteins which are not exposed on the surface. In addition, we investigated the crosslinking of the probe to the membrane phospholipids, using the technique previously developed for the erythrocytes [11].

## Materials and Methods

Isolation of blood platelets, high-resolution two-dimensional polyacrylamide gelelectrophoresis and electro-immuno transfer ('Western blots'), were performed as previously described [7].

*Synthesis of 12-(4-azido-2-nitrophenoxy)-stearoyl[1-$^{14}$C]glucosamine (12-APS-GA).* 12-APS-GA was synthesized essentially as described by Iwata et al. [8]. Briefly, the synthesis involves

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Abbreviations: TLC, thin-layer chromatography; SDS, sodium dodecylsulfate; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; 12-APS-GA, 12-(4-azido-2-nitrophenoxy)stearoyl[1-$^{14}$C]glucosamine.

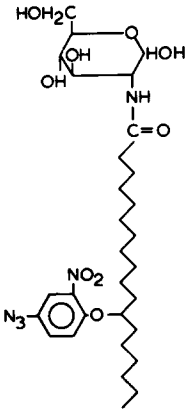


Fig. 1. Chemical structure of the photoactivable probe (12-(2-nitro-4-azidophenoxy)stearoylglucosamine (12-APS-GA).

coupling of 4-fluoro-3-nitrophenylazide to 12-hydroxystearic acid, followed by esterification of the fatty acid analogue with *N*-hydroxysuccinimide. The succinimide ester reacts with the free $NH_2$-group of [1-$^{14}$C]glucosamine (spec. act. 58 Ci/mol) to yield 12-APS-GA. 12-APS-GA and intermediate products were analyzed and purified as described elsewhere [8,11].

*Photolabeling of blood platelets with 12-APS-GA.* To blood platelets (approx. $5 \cdot 10^9$ cells), suspended in 5 ml of buffer containing 10 mM Tris/150 mM NaCl (pH 7.4), 12-APS-GA (4–20 nmol) in ethanol (0.01 ml) was added by injection. The platelet suspension was incubated for 10 min at room temperature or at 37°C, followed by irradiation for 10 min using Philips HPK low-pressure mercury lamp (125 W). A GWV filter with a cut-off below 340 nm was used. At the end of photolysis the platelets were sedimented by centrifugation (10 min at $1000 \times g$). Half of the pellet was resuspended in SDS sample cocktail (0.1 ml), which was used in the SDS-polyacrylamide electrophoresis [7]; the other half was resuspended in 10 mM Tris/150 mM NaCl (pH 7.4) and used for lipid extraction.

In some experiments the platelets after photolysis were resuspended in 10 mM Tris/150 mM NaCl pH 7.4 (5 ml) containing bovine serum albumin (0.1%, w/v), in order to extract the non-coupled 12-APS-GA. Extraction was achieved by incubation for 20 min at 37°C. Upon sedimentation of the platelets, this extraction procedure was repeated two times. The radioactivity removed in each washing step, was determined by liquid scintillation counting.

*Partitioning of 12-APS-GA in the membrane.* In order to establish that 12-APS-GA concentrates in the membrane, the blood platelets incubated with the probe were sedimented by centrifugation (10 min at $1000 \times g$) and the radioactivity in the supernatant was determined. This experiment was performed before and after irradiation of the cells.

*Lipid extraction, separation and analysis.* The lipids were extracted, purified and analyzed as described elsewhere [11]. Briefly, the lipids were extracted according to the procedure of Rose [12]. The lipids, dissolved in 1 ml of chloroform/methanol (95 : 5, v/v), were passed through a column containing 0.75 g silicic acid (Mallinkrodt (C-4)) using acetone (120 ml) and methanol (20 ml) as solvents. The acetone eluent contained the neutral and glyclolipids (e.g., non-coupled probe) and the methanol eluent the phospholipids.

Prior to use, the silicic acid was extensively washed by 1 M ammonia and rinsed with distilled water until the pH was 6 and reactivated at 110°C overnight. Omission of this step sometimes caused hydrolysis of the phospholipids on the columns. The phospholipids were analyzed by TLC (0.25 mm silica gel plates, Merck), using the solvents chloroform/methanol/ammonia/water (90 : 54 : 5.5 : 5.5, v/v) (solvent I) and isopropanol/hexane/water (8 : 6 : 1.5, v/v) (solvent II). Visualization of radioactive phospholipids was performed by autoradiography. Unlabeled phospholipids were visualized by staining with iodine. The labeled phospholipids were identified by comparison with reference phospholipids coupled to 12-APS-GA. The reference phospholipids were prepared as described [11].

*Coupling of 12-APS-GA to membrane proteins.* In order to determine the amount of probe coupled to membrane proteins after irradiation, an aliquot of the packed cells (5% of total) was analyzed by TLC using chloroform/methanol/ammonia (65 : 35 : 5, v/v) as described by Hu and Wisnieski [13]. The plates were scanned for radioactivity using a thin-layer scanner (Panax RTLS-IA). Radioactive spots were scraped from the plate, suspended in 2 ml of water containing BioSolv

(Beckman) and incubated overnight at 50°C. The radioactivity was determined by liquid scintillation counting using Emulsifier (Packard). The amounts of 12-APS-GA coupled to protein was also determined by dissolving the pellet remaining after the lipid extraction, in 2 ml of water containing BioSolv, followed by incubation and counting as described above.

## Results

*Incorporation of photolabel into blood platelets*

Blood platelets were incubated with 12-APS-GA and the binding to the cells and coupling to lipids and proteins was determined (Table I). In the absence of photolysis 94% of the label added was bound to the cells. Subsequent washing of the cells with Tris-buffered saline containing bovine serum albumin removed 90% of the bound probe. After photolysis of the cells, we found that the cells contained 91% of the label added. Under these conditions only 40% of the bound probe was extractable by bovine serum albumin, indicating an extensive coupling to the membrane. In spite of this extensive coupling, a considerable amount of probe (9% of label added) partitions into the aqueous phase upon photolysis (Table I). This suggests that photoactivation yields reaction products of higher polarity as has been noted before [14].

The distribution of covalently linked label between phospholipids and proteins was also determined. The amount of probe in the total lipid fraction was 40–45% of the label bound of which half was associated with the phospholipids (Table I). The remaining protein pellet contained 16% of the label. Coupling to the protein was also analyzed by TLC as shown in Fig. 2. Under the conditions of chromatography the protein of the cell remained at the origin of the plate. This protein fraction contained 15–20% of the radioactivity applied. The bulk of the radioactivity which moves

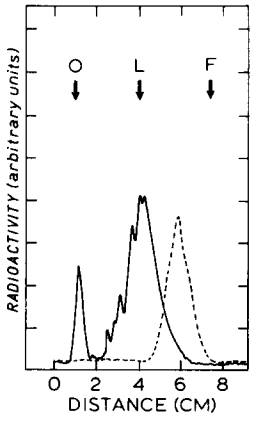


Fig. 2. Radioactive scan of a thin-layer plate of 12-APS-GA coupled to blood platelets. Platelets ($5 \cdot 10^9$ cells) were incubated (10 min, 37°C) in 5 ml Tris-buffered saline and labeled with 12-APS-GA (10 nmol) as described. After irradiation the platelets were sedimented by centrifugation and an aliquot (0.005 ml) containing about 100000 dpm was applied to a silicagel thin-layer plate. The plate was developed using chloroform/methanol/ammonia (60:35:5, v/v). ———, Platelets incubated with 12-APS-GA and irradiated for 10 min. ------, Platelets incubated with 12-APS-GA and not irradiated. 0, origin; L. lipid; F, front.

TABLE I

INCORPORATION AND COUPLING OF 12-APS-GA TO THE PLATELET MEMBRANE

Blood platelets were incubated with 12-APS-GA ($5 \cdot 10^6$ dpm) and the partitioning of the label was determined as described in Materials and Methods. After photolysis, the uncoupled probe was extracted from the platelets using 10 mM Tris/150 mM NaCl (pH 7.4), containing bovine serum albumin (BSA) (0.1%, w/v) followed by lipid extraction according to Rose [12]. The lipid fraction was separated on a silicic acid column by using acetone and methanol as solvents. The radioactivity in the acetone fraction, methanol fraction (phospholipids) and the residual protein material was determined.

| Sample | Intact cell (% of label added) | % of label bound | | | |
|---|---|---|---|---|---|
| | | BSA extractable | Acetone fraction | Phospho-lipids | Proteins |
| Irradiated | 91 | 40 | 22 | 22 | 16 |
| Control | 94 | 90 | n.d. | 0 | 0 |

away from the origin, represents the probe both non-coupled and coupled to the lipids. In the absence of irradiation no radiactivity was detected at the origin. Moreover, the $R_F$ of the non-irradiated probe was greater than that of the photolyzed probe. This difference in $R_F$ may reflect the more polar character of the reaction products.

*Patterns of membrane proteins labeled with 12-APS-GA*

The membrane proteins labeled with 12-APS-GA were analyzed by two-dimensional electrophoresis according to O'Farrell with a pH gradient of 4.5 to 6.5 and 7.5% polyacrylamide gel after reduction in the second dimension. The autoradiogram (Fig. 3A) provides a pattern of membrane proteins which is to a large extent similar to the pattern obtained by ectolabeling (Fig. 3b).

The main difference between the two labeling patterns was the following: three 'new' proteins were seen, one at 67 kDa, a second at 50 kDa, and a third at 45 kDa. The glycoproteins Ia and Icα were not observed in the photolabelling pattern, whereas IIa, Ibα, IIbα, III and IV were clearly labeled. The non-reduced/reduced patterns (Fig. 4) indicated labeling of thrombospondin, fibrinogen and albumin, but this labeling was weak and probably not responsible for the spots at 67 and 50 kDa in the O'Farrell gels. Spots corresponding to these molecular weights were observed near the diagonal on the non-reduced/reduced gels. No labeling was observed of myosin, tubulin, or α-actinin with either labeling technique. Labeling was observed at the location of actin but whereas Coomassie blue labeling gave a homogenous spot, radiolabelling gave several spots on the acidic site, indicating that not actin but another protein was responsible for this labeling. The labeling of the Ibβ and IIbβ was stronger than that of the corresponding α subunits.

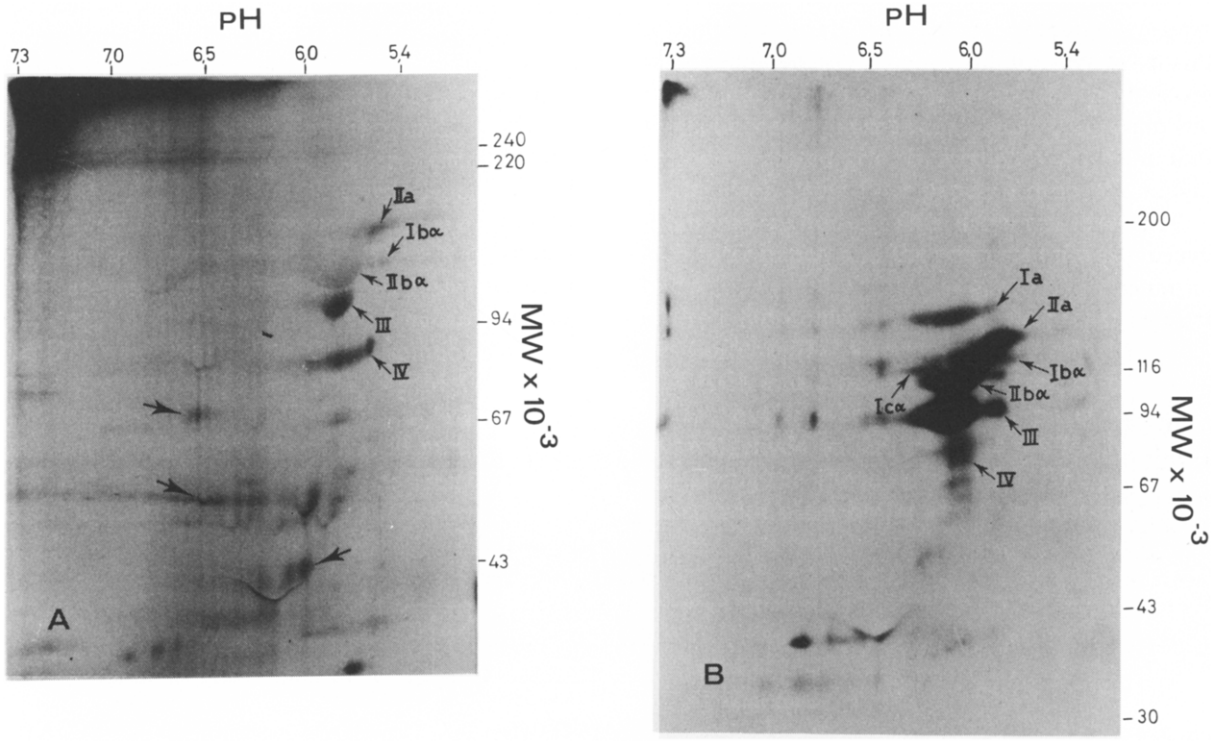


Fig. 3. Autoradiogram of high resolution two-dimensional gel of labeled platelet membrane proteins. (A) 7.5% SDS-polyacrylamide gel of reduced membrane proteins labeled with 12-APS-GA. The 'new' proteins observed are indicated with arrows. (B) 3–30% SDS-polyacrylamide gradient gel of reduced membrane proteins labeled with lactoperoxidase iodination.

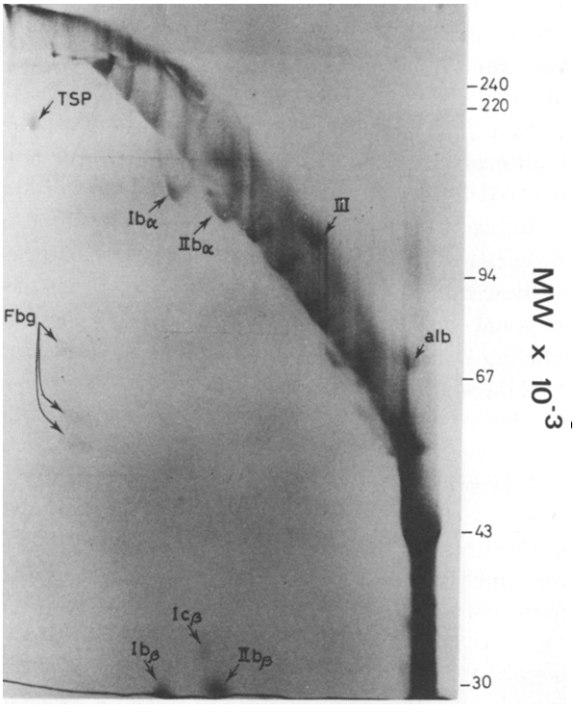


Fig. 4. Autoradiogram of a non-reduced/reduced two-dimensional gel of platelet membrane proteins labeled with 12-APS-GA. Electrophoresis of non-reduced samples was from left to right. Electrophoresis after reduction was from the top downward.

*Labeling of phospholipids*

Phospholipids have been shown to the assymmetrically distributed between the two halves of the plasma membrane of blood platelets, with phosphatidylserine concentrated in the inner monolayer [15]. In order to obtain evidence for 12-APS-GA being restricted to the outer leaflet of the bilayer [9,10], we studied the labeling pattern of the various lipids on TLC using solvents I and II. As shown in Figs. 5A and 5B, PE, PS and PC were extensively labeled (lane 5). For identification reference phospholipids, crosslinked to 12-APS-GA, have been included consisting of PE/PC (lane 1), PI/PC (lane 2), PS/PC (lane 3S) and PC and sphingomyelin (lane 4). In both solvent systems contaminating radiolabeled components were present, which mostly ran ahead of PE and reflect the incomplete removal of non-covalently coupled probe by prior chromarography on the silicagel column. The identification of PS is complicated by the fact that PI has a similar $R_F$ value. On the other hand, PI is a minor component in the plasma membrane which is also supposed to be located on the cytoplasmic side [16]. The distinct labeling of PS strongly suggests transbilayer movement of the probe before and/or during photolysis.

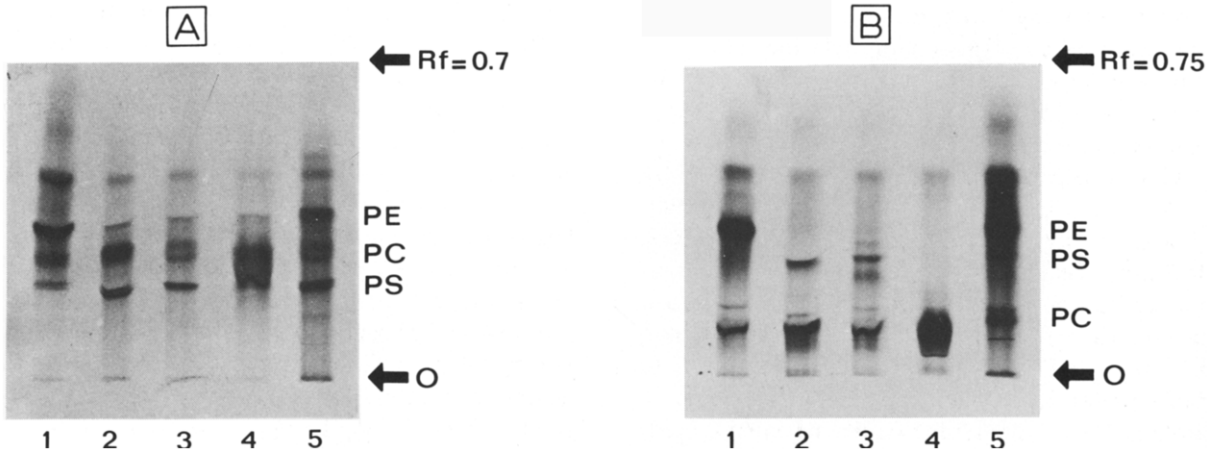


Fig. 5. Autoradiograms of platelet phospholipids labeled with 12-APS-GA upon TLC. Platelets were incubated with 12-APS-GA (10 nmol) at room temperature and irradiated for 10 min. The phospholipids were extracted, fractionated and chromatographed as previously described, using solvent I (Fig. 5A) or solvent II (Fig. 5B). Reference phospholipids consisted of PE and PC (lane 1), PI and PC (lane 2), PS and PC (lane 3) and PC and sphingomyelin (lane 4), coupled to 12-APS-GA. Phospholipids of intact platelets labeled by 12-APS-GA are shown in lane 5.

## Discussion

The photoactivable glycolipid probe, 12-APS-GA, was developed by Wisnieski and co-workers [9], to label integral membrane proteins from within the hydrophobic core of the membrane. The design of the probe (see Fig. 1) ensures an anchoring of the nitrene-generating group in the membrane core by its attachment to the fatty acyl chain, while the carbohydrate moiety is meant to restrict the probe to the outer leaflet of sealed membrane systems [8–10]. In the case of coliphage M13 coat protein reconstituted in PC vesicles, evidence was provided that this probe coupled to the integral membrane peptides and not to the amino acid residues exposed on the outside of the bilayer [13,17]. Here we have used 12-APS-GA to identify the integral membrane proteins of human blood platelets. In a previous study we have analyzed the proteins of the plasma membrane by ectolabeling of intact blood platelets [5]. Comparison of the labeling patterns demonstrated that labeling with 12-APS-GA yielded three 'new', not previously identified, proteins of 67, 50, and 45 kDa. Since ectolabeling will be restricted to adsorbed proteins and the external peptide fragments of intrinsic membrane proteins, we infer that the 67, 50, and 45 kDa proteins are intrinsic to the membrane shielded from the outside medium. The 67 kDa protein is probably identical to the 70 kDa protein found by Rotman et al. [18] with photoactivable probe [$^{125}$I]iodonaphthylazide. This protein was present in increased concentrations in pseudopods. The 50 and 45 kDa proteins were not observed with this probe.

Glycoprotein Ia and Ic$\alpha$ were not labeled by 12-APS-GA. One should realize, however, that photoactivation of membrane-lodged 12-APS-GA does not necessarily lead to a labeling of all available intrinsic proteins. In general, the photoreactive intermediate of aryl azides have relatively long lifetimes and are selective for coupling to nucleophiles like tyrosine, serine or cysteine in proteins [19,20], or double bonds in phospholipids [21]. This implies that the amino acid composition of the membrane-embedded protein segment is a determining factor in the final labeling pattern obtained.

Glycoprotein V was not clearly observed in the high resolution two dimensional gel (Fig. 3), but it is probably present in the non-reduced-reduced gel (Fig. 4). The $\beta$-subunits of glycoprotein Ib and IIb were more extensively labeled than the corresponding $\alpha$-subunits. This may indicate that the $\beta$-subunits are to a greater degree membrane-embedded, but differences in amino acid composition of the membrane embedded segment have been responsible. As in previous studies with labeling with lactoperoxidase iodination, we found some labeling of fibrinogen and thrombospondin. The most likely explanation for this is, that platelet washing has caused some release of these proteins, which stick to the platelet membrane after activation.

In a previous study with intact human erythrocytes, 12-APS-GA was found to yield an extensive labeling of intrinsic membrane proteins [11]. An extensive labeling of a band coinciding with band 3 protein was noted as well as a distinct labeling in the region where the cytoplasmically oriented membrane proteins (i.e. 6 and 7) collect. The labeling of the latter class of proteins was unexpected as, in intact erythrocytes, 12-APS-GA was presumed to be restricted to the outer leaflet of the membrane [9,10]. Analysis of the platelet phospholipid fraction after photolysis indicated that, in addition to PC and PE, PS was distinctly labeled by 12-APS-GA (see Fig. 5). In studies using phospholipases and 2,4,5-trinitrobenzenesulfonate, PS was found to be mainly residing in the inner leaflet of the platelet plasma membrane [15,22]. Hence, we infer from our lipid labeling data that most likely transbilayer movement of 12-APS-GA occurs. This is in agreement with our observations on intact erythrocytes [11].

In contrast to Hu and Wisnieski [13], we have found that coupling of 12-APS-GA to dimyristoyl-PC in vesicles is very limited (unpublished observation). Our observations agree with the notion that aryl nitrenes do not easily insert into C-H bonds [20,23]. We propose that the observed extensive labeling of the platelet phospholipid fraction (see Table I) reflects an addition of the photogenerated nitrene to fatty acid double bonds [11,21]. Labeling of both PC, PE and PS, suggests a random distribution of 12-APS-GA in the membrane (Fig. 5). Recently, Delclos et al. [24]. made use of a phorbol ester containing an aryl azido

moiety in order to identify the receptor for this tumour promotor in mouse brain membranes. Interestingly, PS and PE were found to be selectively labeled, suggesting that these phospholipids formed an intricate part of the receptor.

In general, application of hydrophobic photolabels to membrane studies has met with many complications and, due to the uncertainty of probe localization, has yielded results of limited significance. The present study provides proof that 12-APS-GA is a useful probe for identification of intrinsic membrane proteins. The recent developments of hydrophobic photolabels with an affinity for distinct membrane proteins (e.g., receptors) [25–27] will further advance our understanding of the molecular architecture of biological membrane.

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