

Labeling of human erythrocyte membrane proteins by photoactivatable radioiodinated phosphatidylcholine and phosphatidylserine

A search for the aminophospholipid translocase

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We have synthesized radioiodinated photoactivatable phosphatidylcholine ($^{125}\text{I-N}_3\text{-PC}$) and phosphatidylserine ($^{125}\text{I-N}_3\text{-PS}$). After incubation with red blood cells in the dark, the labeled PC could be extracted but not the corresponding PS molecule, indicating that the latter was transported by the aminophospholipid translocase, but not the former. When irradiated immediately after incorporation, $\text{N}_3\text{-PS}$, but not $\text{N}_3\text{-PC}$, partially blocked subsequent translocation of spin-labeled aminophospholipids. Analysis of probe distribution by SDSpolyacrylamide gel electrophoresis revealed that $^{125}\text{I-N}_3\text{-PS}$ labeled seven membrane bound components with molecular masses between 140 and 27 kDa: one (or several) of these components should correspond to the aminophospholipid translocase.

Phospholipid flip-flop; Nitrene; Erythrocyte; Spin-label; ESR

1. INTRODUCTION

Phospholipids are asymmetrically distributed between the two leaflets of the human erythrocyte membrane: phosphatidylcholine and sphingomyelin are the major components of the outer layer, while the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are located preferentially on the inner layer [1,2]. This stable asymmetry seems to be produced and main-

tained by a specific ATP requiring transport protein, the existence of which was first postulated in our laboratory from ESR experiments [3]. In subsequent work, we have shown that PS and PE use the same carrier system which can be inhibited by protein reagents such as *N*-ethylmaleimide or ions such as calcium and vanadate [4]. Several laboratories have now shown that when exogenous aminophospholipids are introduced into the outer leaflet of the erythrocyte membrane, they are rapidly transported to the inner leaflet providing the cells contain ATP [5,6]. The question remains to identify which protein(s) of the erythrocyte surface is (are) involved. In a recent study Schroit et al. [7] introduced a radioiodinated photoactivatable phosphatidylserine molecule in human erythrocytes. Their probe preferentially labeled 30 kDa protein which may be responsible for the phosphatidylserine translocation. We have syn-

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Abbreviations: BSA, bovine serum albumin; ESR, electron spin resonance; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography

thesized radioiodinated photoactivatable phospholipids slightly different from the ones used by Schroit and collaborators. Here we show that the phosphatidylserine analogue used in our experiments is an inhibitor of aminophospholipid translocation after irradiation. It labels several bands on gel electrophoresis, leaving several possible attributions for the aminophospholipid translocase.

2. MATERIALS AND METHODS

2.1. Synthesis

The synthesis of photoactivatable phospholipids (Fig. 1) was adapted from [8] and carried out in the dark. 150 μ mol of 4-azido-2-hydroxybenzoic ester of *N*-hydroxysuccinimide (Pierce) and 155 μ mol of β -alanine ethyl ester hydrochloride (Serva) were dissolved in 6 ml chloroform containing 45 μ l triethylamine and stirred for 30 min. The ethyl *N*-(2-hydroxy-4-azidobenzoyl)-3-aminopropionate formed was purified by TLC and transformed quantitatively into the corresponding acid by an overnight incubation in 1.5 ml dioxane/2.7 ml sodium hydroxide solution (2 mol per liter of water). After acidification to pH 3, the reaction mixture was extracted three times by chloroform. The overall yield was better than 70%. The esterification of this acid onto lysophosphatidylcholine to yield photoactivatable PC was performed according to Samuel et al. [9]. Photoactivatable PC was transformed into photoactivatable PS by the phospholipase D-catalysed base-exchange reaction as described by Comfurius and Zwaal [10]. Iodination of the photoactivatable phospholipids was carried out as follows: 5 μ g N_3 -lipid in 20 μ l chloroform were mixed with 20 μ l $Na^{125}I$ (2 mCi, carrier-free; Amersham); iodination was started by

addition of 10 μ l of 5 mg/ml chloramine T in 0.5 M phosphate buffer, pH 7.5. After 30 s vortexing, the reaction was stopped with 40 μ l sodium metabisulfite (50 mg/ml)Kl (20 mg/ml) in 0.05 M phosphate buffer. The ^{125}I - N_3 phospholipid was then extracted four times with 250 μ l chloroform and analysed by TLC on silica gel plates and by autoradiography. When necessary, the phospholipid was purified on CM-52 cellulose column according to [10].

2.2. Photoactivation

Freshly drawn erythrocytes were washed five times in 130 mM NaCl, 20 mM phosphate buffer, pH 7.4, and treated by 5 mM di-isopropyl-fluorophosphate to inhibit phospholipase activities. They were then brought to 50% hematocrit and incubated in the dark with the photoactivatable phospholipids for either 2 or 60 min at 37°C. Cell suspension was then diluted 20 times with ice-cold buffer, transferred into a Petri dish and placed on ice 10 cm below a 30 W, 312 nm-UV lamp for 10 min. Cells were then washed twice in NaCl-phosphate buffer.

2.3. Aminophospholipid translocase-binding assay

The activity of the phospholipid translocase was measured by the spin-label assay [3,4]. Briefly spin-labeled PS or PE were introduced at 4°C in erythrocytes and their translocation towards the inner leaflet monitored by measurement of the fraction of spin-labels resistant to externally added ascorbate. In order to determine if the photoactivatable lipids bind to the translocase, two types of experiments were run. First, the outside-inside distribution of spin-labeled PE was assessed in the presence of non-irradiated N_3 -PS or N_3 -PC. Secondly, erythrocytes supplemented with either N_3 -PS or N_3 -PC (2% of membrane phospholipids) were incubated at 37°C (for 2 or 30 min), and irradiated. Samples were subsequently washed twice with 1% BSA in phosphate buffer and twice in buffer alone, and spin-labeled PS transmembrane reorientation measured. Controls without photoactivation were run in parallel.

2.4. Analysis of photolysis products

White ghosts were prepared from washed, UV-irradiated erythrocytes by repetitive washings in 5

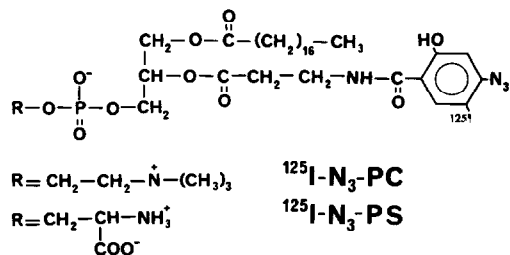


Fig. 1. Structure of ^{125}I - N_3 -PC and ^{125}I - N_3 -PS.

mM phosphate buffer and solubilised in electrophoresis sample buffer containing SDS and β -mercaptoethanol. Electrophoresis was performed using Laemmli's discontinuous system [11], with a 5% acrylamide stacking gel and a 10% acrylamide separating gel, at a constant current intensity of 40 mA per gel. Gels were then stained, dried and autoradiographed for two weeks.

3. RESULTS

3.1. Inhibition of aminophospholipid translocation

Non-irradiated N_3 -PC in the erythrocyte membrane did not impair the transmembrane relocation of spin-labeled PE, which shows the absence of competition between the spin-labeled PE and the photoactivatable PC. By contrast, N_3 -PS inhibited the relocation of spin-labeled PE (not shown) and, thus competes with PE for the

translocase. From the decrease in the initial velocity of PE reorientation, we inferred an apparent affinity of N_3 -PS for the aminophospholipid translocase comparable to that of spin-labeled PE, i.e. $\approx 50 \mu\text{M}$ [4]. Photoactivation of N_3 -PC embedded in the red cell membrane did not affect reorientation kinetics assayed with spin-labeled PS (fig.2). On the other hand, UV irradiation of N_3 -PS, following 2 min preincubation, reduced the initial velocity of spin-labeled PS to $72 \pm 4\%$ of the control; after 30 min preincubation the inhibition was less pronounced, leaving $87 \pm 3\%$ of the velocity. Control experiments run in the conditions of photoactivation but without actual irradiation, showed that subsequently to the washing with BSA, there was no inhibition of the aminophospholipid reorientation. Thus, N_3 -phospholipids incubated with cells for a short period of time are fully extractable.

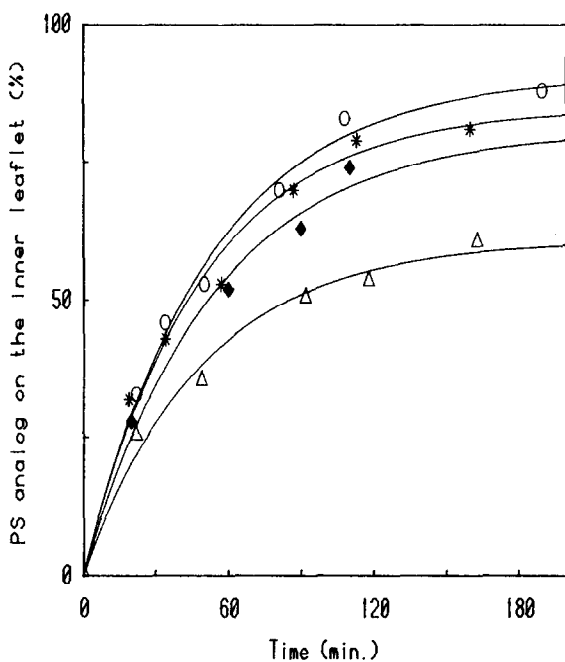


Fig.2. Kinetics of transmembrane relocation at 4°C of spin-labeled PS in erythrocytes irradiated in the absence of N_3 -phospholipids (\circ), in the presence of N_3 -PC (\star), in the presence of N_3 -PS after a 2 min incubation (Δ) or a 30 min incubation and albumin extraction (\diamond). Transmembrane relocation was assayed as described in [3,4].

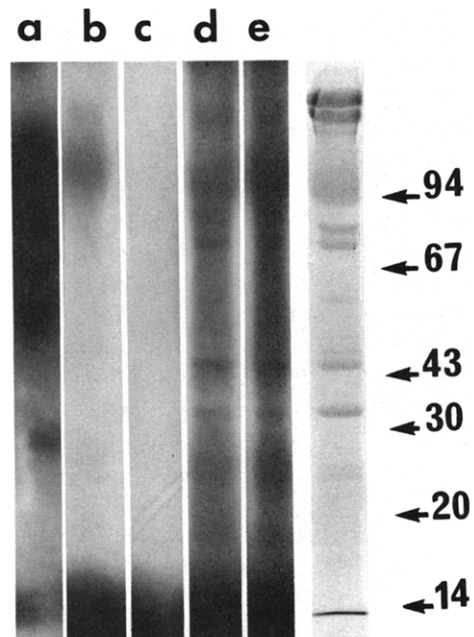


Fig.3. Autoradiographs obtained after electrophoresis of photoaffinity-labeled erythrocyte membranes (see section 2). Gels are stained with Coomassie blue (right lane) and autoradiographed. Lanes: a and b, two different concentrations of ^{125}I - N_3 -PC; c, ^{125}I - N_3 -PS without irradiation; d and e, ^{125}I - N_3 -PS illuminated after 2 min or 60 min incubation at 37°C . Arrows indicate the migration of molecular mass marker proteins.

3.2. Membrane labeling

Fig.3 shows the electrophoretic pattern of Coomassie blue-stained membrane proteins and the autoradiographic patterns obtained under various conditions. In the absence of irradiation, no spot can be detected except at the dye front (lane c). The pattern observed after photoactivation following a 2 min incubation at 37°C is different for $^{125}\text{I-N}_3\text{-PC}$ (lanes a and b) and $^{125}\text{I-N}_3\text{-PS}$ (lane d): several bands are well individualized with the latter probe while the former one indicates an efficient coupling of undistinct pattern. If cells incubated with less $^{125}\text{I-N}_3\text{-PC}$ are analysed (lane b), one can see that the major component labeled comigrates with band 3 protein (nomenclature according to Steck [12]). UV-light irradiation of cells which had been incubated 60

min at 37°C in the presence of $^{125}\text{I-N}_3\text{-PS}$ led to a pattern (lane e) which closely resembled that obtained after a short incubation (lane d), with the exception of a more intense spot comigrating with proteins of $M_r \sim 27\,000$. This is more obvious on the profiles obtained by autoradiograph scanning (fig.4): trace A shows the labelling by $^{125}\text{I-N}_3\text{-PS}$ after a 2 min incubation while trace B was obtained after a 60 min incubation. If, prior to incubation with $^{125}\text{I-N}_3\text{-PS}$, cells were incubated and irradiated in the presence of an excess of cold $\text{N}_3\text{-PC}$ or cold $\text{N}_3\text{-PS}$, the autoradiography profiles obtained are different as shown in fig.4C and D. A pre-treatment of erythrocytes by 1 mM *N*-ethylmaleimide as described in [4] abolishes all subsequent labeling by $^{125}\text{I-N}_3\text{-PS}$, except for components migrating at the dye front (trace E, fig.4). If, after a 60 min incubation at 37°C, cells were washed with BSA prior to UV illumination, no labeling by $^{125}\text{I-N}_3\text{-PC}$ remained while labeling by $^{125}\text{I-N}_3\text{-PS}$ exhibited a profile resembling that of trace B.

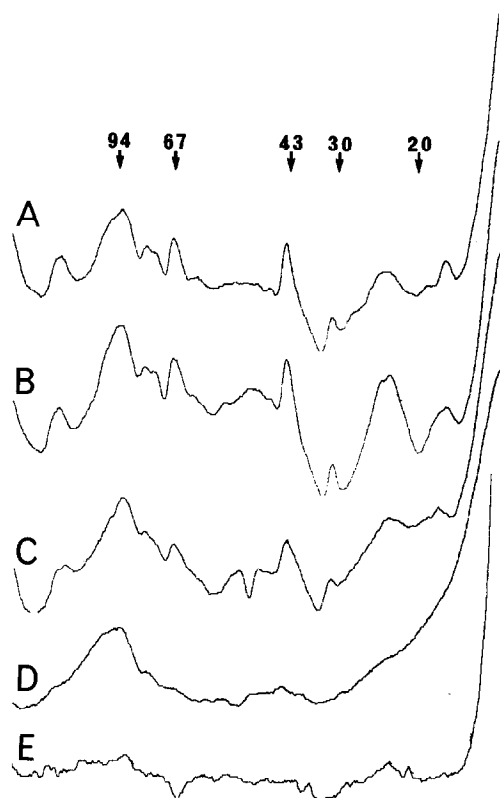


Fig.4. Density profiles of autoradiographs: erythrocytes incubated with $^{125}\text{I-N}_3\text{-PS}$ for 2 min (A) or 60 min. (B) at 37°C before irradiation, or pretreated with an excess of cold $\text{N}_3\text{-PC}$ (C) or cold $\text{N}_3\text{-PS}$ (D) prior to addition of $^{125}\text{I-N}_3\text{-PS}$; erythrocytes incubated with *N*-ethylmaleimide before addition of $^{125}\text{I-N}_3\text{-PS}$ (E).

DISCUSSION

In this paper, we have described the synthesis of radioiodinated photoactivatable phospholipids ($^{125}\text{I-N}_3\text{-PC}$ and $^{125}\text{I-N}_3\text{-PS}$), which we have used for the differential labeling of human erythrocyte membrane proteins. Both lipids incorporated spontaneously into the outer leaflet of erythrocytes from which they could be extracted by washing with BSA. However, if $\text{N}_3\text{-PS}$ was allowed to incubate in red cells, prior to BSA extraction, it remained in the erythrocytes, suggesting a translocation towards the inner leaflet by the aminophospholipid translocase. Similar observations were made by Schroit et al. [7], using different photoactivatable phospholipids; these authors interpreted their data as being indicative of a 'specific immobilisation' of the PS derivative in the cells' outer leaflet. That the $\text{N}_3\text{-PS}$ selectively interacts with the aminophospholipid translocase and not the $\text{N}_3\text{-PC}$, is also indicated by the experiments where the photoactivatable lipids are coupled to the proteins by irradiation. Indeed, after UV irradiation, $\text{N}_3\text{-PS}$, and not $\text{N}_3\text{-PC}$, partially inhibited the activity of the transport enzyme as judged from subsequent assays conducted with spin-labeled aminophospholipids (fig.2). The in-

hibition very likely indicated a covalent binding of N_3 -PS to the aminophospholipid translocase. Note that if N_3 -PS was incubated with the membranes before irradiation, hence was allowed to reach the inner leaflet before chemical coupling, then no inhibition took place. This suggests that the affinity of phosphatidylserine derivatives to the translocase, from the inner layer, is low.

As can be seen from the autoradiographs (fig.3), ^{125}I - N_3 -PC and ^{125}I - N_3 -PS label differently the proteins of the red blood cell membrane. First, as already quoted by Schroit et al. [7], there is a higher yield of binding with the PC derivative than with the PS derivative. If irradiation is carried out under condition of weak binding, the PC derivative labels mainly band 3 protein, which probably reflects solely the fact that band 3 accounts for approximately 50% of the total intrinsic proteins of erythrocyte membranes [13]. ^{125}I - N_3 -PS migrates with several bands which are resolved in the scans shown in fig.4A. We detect seven major membrane components which are migrating as the following proteins (according to the nomenclature of Steck [12]): unknown (~140 kDa), band-3 (95 kDa), PAS-1 (80 kDa), PAS-4 (67 kDa), band 5 or PAS-2 (44 kDa), band 6 (32 kDa) and PAS-3 or band 7 (27 kDa). An additional band seen at 17 kDa corresponds most likely to a hemoglobin polypeptide. Incubation at 37°C which allows some ^{125}I - N_3 -PS to be transported into the inner membrane leaflet, is accompanied by an increased labelling of the 27 kDa peptide. Either this polypeptide is easier to label from the inside of the cell or we detect two comigrating proteins, one of which being localized on the cytoplasmic face. Since the inhibitory property of N_3 -PS on the aminophospholipid translocation is lost when membranes are irradiated after incubation at 37°C, this band does not appear to be a good candidate for the the protein being searched. This band would correspond to the major band labeled by Schroit et al. [7] with their photoactivatable PS, since, according to these authors, it comigrates with band 7.

Binding experiments carried out with prior irradiation in the presence of cold N_3 -PC or cold N_3 -PS (fig.4C and D) show that ^{125}I - N_3 -PS: (i) does not compete for binding with N_3 -PC; but (ii) competes with N_3 -PS. This latter conclusion proves that the iodinated derivative is chemically

equivalent to the noniodinated molecule and that the number of phosphatidylserine binding sites is limited, except perhaps on band 3 protein. Finally preincubation with *N*-ethyl-maleimide (fig.4E) clearly confirms that the photoexcited nitrenes have a preference for SH groups [14]. Thus the distribution of radiolabel among the components of red blood cells is probably sensitive to the chain length of the fatty acid which bears the nitrene residue. It is therefore not surprising that our autoradiograph patterns are not identical to the ones published recently by Schroit et al. [7]. In fact the two investigations are quite complementary. Definitive assignment of the aminophospholipid translocase will await for a different type of labeling.

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