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Erythrocyte band 3 protein strongly interacts with phosphoinositides

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Abstract

85% of the phosphorus coisolated with band 3 protein during separation of the intrinsic proteins of the human erythrocyte membrane by zonal electrophoresis in high concentrations of acetic acid was found to be derived from phosphoinositides, mainly phosphatidylinositol 4,5-bisphosphate. When native band 3 protein and pyrene-labelled phospholipids were present in micelles of the nonionic detergent nonaethyleneglycol lauryl ether, strong resonance energy transfer was observed between the tryptophan residues and phosphatidylinositol 4,5-bisphosphate and, to a smaller degree, phosphatidylinositol 4-phosphate. We conclude that band 3 protein strongly interacts with phosphoinositides, in particular with phosphatidylinositol 4,5-bisphosphate.

Key words: Band 3 protein; Phosphoinositide; Protein–lipid association; Erythrocyte membrane

1. Introduction

Specific associations between intrinsic membrane proteins and phosphoinositides have attracted much attention, due to their known or potential role in the regulation of various membrane functions [1,2]. They have also been observed with proteins of the erythrocyte membrane [3]. In the latter system, the glycophorins represents the most intensely studied proteins. Their association with phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) was first inferred from the coisolation of the two types of molecules during purification of the protein [4,5] and later supported by other findings [6] (but also challenged by another one [7]). Strong protein–phosphoinositide interactions were also observed with another red cell membrane protein, the Ca^{2+}-transport ATPase [8]. Similar findings concerning the most abundant intrinsic protein of the erythrocyte membrane, band 3, have not been reported. However, prompted by previous observations that certain preparations of human band 3 protein contain phosphorus not extractable by standard methods of lipid removal [9], we have now found that also band 3 strongly interacts with phosphoinositides. This result is based on two lines of evidence: (1) the identification of the tightly bound lipids in the band 3 samples as phospho-inositides; and (2) the demonstration of strong resonance energy transfer between native band 3 and pyrene-labelled PIP_2 and PIP in micelles of a nonionic detergent.

2. Materials and methods

2.1. Isolation of human band 3 by zonal electrophoresis in acetic acid/water/sucrose mixtures and identification of bound phospholipids

The solubilization and purification were performed according to [9]. The organic solvent and the sucrose were removed by dialysis, and the protein was concentrated by isoelectric precipitation and redissolved at pH 9 [9,10]. Afterwards, approx. 1.0 mg of SDS per mg of protein was added, and the protein was freeze-dried. Two protein batches were analyzed (containing 58 mg (A) and 73 mg (B) of band 3, respectively, as quantified by absorbance measurements [10]). During isolation of another batch of 26 mg band 3 (C), 15 mg of a crude phosphoinositide fraction from bovine brain (Sigma) were added to the sample before electrophoresis.

To identify protein-bound lipid, samples of 10 mg (batches A and B) or 3 mg (batch C) of the freeze-dried protein were dissolved in 1 ml of water and extracted with 9 ml of acidic chloroform/methanol [11,12]. Aliquots of the washed lipids were subjected to TLC on 10 × 10 cm high performance TLC plates (Merck) in chloroform/methanol/4 M NH_4OH [13] or chloroform/acetone/methanol/acetic acid/water [14]. PIP_2, PIP and PI used as standards were purified from bovine brain and were a gift of Dr. K. Hayashi, Ibaraki University, Japan. Quantitation of the lipids was performed according to [14], scanning being done on a Camag TLC densitometer at 450 nm.

2.2. Fluorescence spectroscopic studies on mixtures of pyrene-labelled phospholipids and detergent-solubilized band 3

sn-2-(Pyrenyldecanoyl)-PI (pyrPI) was synthesized from yeast PI according to Somerharju et al. [15,16]. PyrPIP and pyrPIP$_2$ were synthesized from pyrPI using partially purified PI and PIP kinase preparations as described by Gadella et al. [17]. PyrPC was synthesized from egg yolk PC [16], and pyrPS was obtained from pyrPC by transphosphatidyla-

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Abbreviations: $C_{12}E_9$, nonaethyleneglycol lauryl ether; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phospatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; SM, sphingomyelin.

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tion catalysed by phospholipase D [18]. PyrPE, pyrPG and pyrSM were purchased from Molecular Probes, Inc.

Human band 3 was solubilized and purified in solutions of non-aethyleneglycol lauryl ether ($C_{12}E_9$) (Sigma or Fluka), using a modified version [19] of the method of Yu and Steck [20]. After gel filtration [19] and subsequent dilution of the protein to a concentration of 1–15 μg/ml the buffer was 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.01% $C_{12}E_9$. Then, up to 0.05 volumes of a solution of pyrene-labelled lipids [8,17] (in ethanol/dimethyl-sulfoxide 3 : 1 v/v); lipid concentration 1–60 μM [8]) were added to the sample. Before fluorescence measurements, the protein/detergent/lipid mixture was incubated overnight at 1°C.

Measurements of resonance energy transfer were performed on a SLM-Aminco DMX-1000 or a Perkin-Elmer LS 50 spectrofluorometer. The cuvette holder was held at 1° C. The excitation wavelength was set at 295 nm. The efficiency of energy transfer (E) was calculated from the fluorescence intensities (at 335 nm) of the donor, tryptophan, in the presence (F_{da}) and absence (F_d) of the acceptor, pyrene, by the equation [21]:

$$E = 1 - F_{da}/F_d \quad (1)$$

The absorbance of all samples was below 0.02 in the wavelength range used.

3. Results

3.1. Band 3-bound phospholipids

Band 3 isolated by zonal electrophoresis in an acetic acid/water/sucrose gradient contains (5.1 ± 0.4) mol of phosphorus per mol of protein [9]. The corresponding compounds were made extractable, by acidic chloroform/methanol, by addition of SDS to the protein solution before freeze-drying. Applying the methodology described, approximately 85% of the total phosphorus bound to band 3 could be shown to originate from phosphoinositides (the remainder, which runs as a sharp band between PIP and PI, could not be identified but may represent lyso-PI). The composition of the extracted phosphoinositides is given in Table 1, together with the corresponding data found with a band 3 preparation to which a mixture of phosphoinositides had been added after solubilization of the protein. The following results emerge from the data:

(1) Approx. 64% of the total phosphorus content of the protein originates from PIP_2, the stoichiometry PIP_2/band 3 being close to 1.0.
(2) On a molar basis, PI is virtually as abundant in the sample as PIP_2 (We have ruled out, by control experiments with PIP_2 or the crude phosphoinositide batch, that the PI recovered is formed from PIP_2 by acid hydrolysis during the isolation procedure). On the other hand, the molar ratio of PIP to band 3 is only approximately 0.25.
(3) The relative molar abundancy of the three phosphoinosites in the samples is similar to that reported for isolated erythrocyte membranes [22–24].
(4) The phosphoinositide binding to band 3 described does not represent saturation of the binding sites.

In contrast to PIP_2 and PIP, PI could already be extracted in neutral chloroform/methanol.

Interestingly, during isolation of band 3 by zonal electrophoresis virtually no phospholipid migrates with the glycophorins (which are clearly separated from band 3) [9].

Table 1
Phosphoinositide content of the isolated band 3 (in mol per mol of protein)

Prep.	PIP_2	PIP	PI	n^+
A	1.10 ± 0.15	0.30 ± 0.15	1.05 ± 0.20	5
B	1.2 ± 0.4	0.20 ± 0.05	1.05 ± 0.10	7
C*	4.0 ± 0.6	1.3 ± 0.3	3.2 ± 0.4	7

^+n: number of determinations.
*Sample C was isolated in the presence of added phosphoinositides.

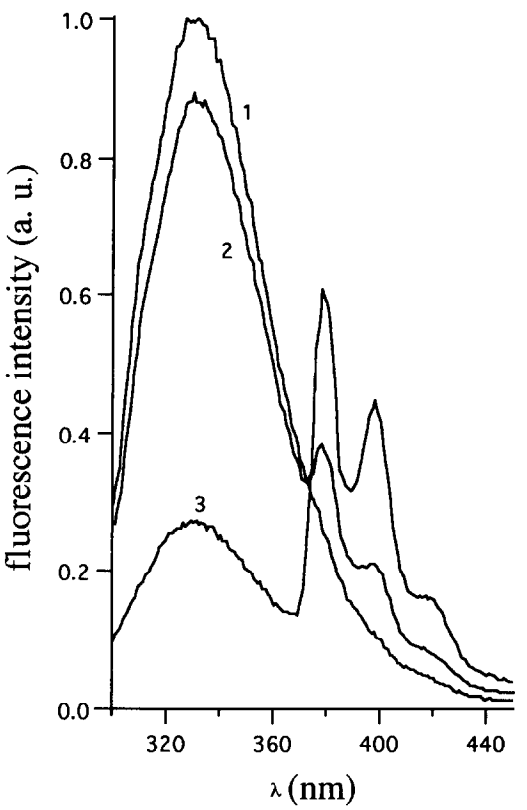

Fig. 1. Fluorescence emission spectra of solutions of band 3 protein (150 nM) in the absence of lipid (1) and in the presence of 250 nM pyrene-labelled PC (2) or PIP_2 (3). The spectra were corrected for the Raman peak of water.

3.2. Energy transfer measurements

Band 3 protein in micelles of $C_{12}E_9$, as isolated by us, is in a native state (as judged from reconstitution experiments [25] and binding studies [26]) and is virtually free of phospholipids [19,20]. When pyrene-labelled phospholipids are inserted into band 3/$C_{12}E_9$ micelles, irradiation of the samples at 295 nm leads to emission of light at the characteristic emission wavelengths of pyrene

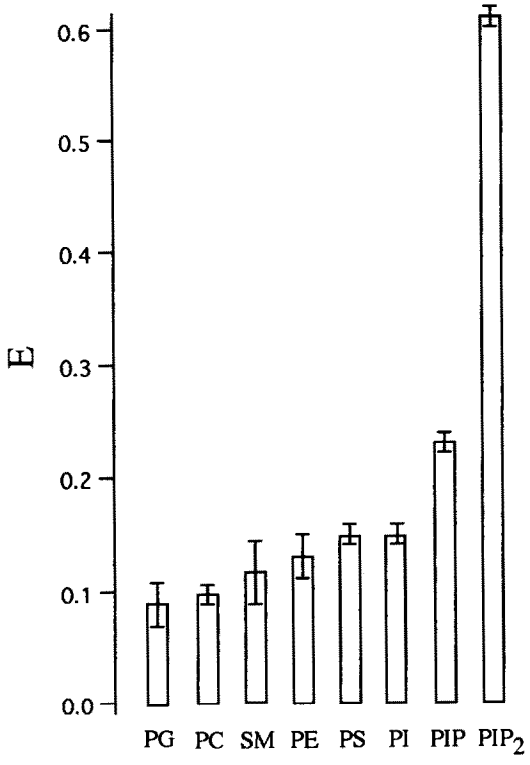

Fig. 2. Efficiency E of energy transfer between solubilized band 3 protein and different pyrene-labelled phospholipids. Lipid concentration, 200 nM; protein concentration, 150 nM. The figure combines data from two series of measurements (I: PIP_2, PIP, PI, PC; II: all other lipids and PC), using different protein preparations. The *E*-values from series II were normalized to that for PC(I).

monomers. At the same time, tryptophan emission is much weaker than without the lipid (Fig. 1). Control experiments with unlabelled lipids showed no effect on the donor emission spectra. Thus, the data of Fig. 1 demonstrate a close association of band 3 with the lipids in the micelles that leads to resonance energy transfer [21]. The efficiency of energy transfer, as characterized by Equation (1), was strongly dependent on the chemical structure of the phospholipid used. As shown in Fig. 2, by far the most efficient transfer was observed with PIP_2, followed by PIP and PI. However, the effect of PI on E was very similar to that of other phospholipids.

Our results closely resemble those found with the Ca^{2+}-transport ATPase of the human erythrocyte membrane by the same technique [8].

4. Discussion

The experiments described above show that two different preparations of isolated band 3 protein strongly interact with phosphoinositides. Lipid analyses on band 3 purified in high concentrations of acetic acid indicate a 1:1 association with PIP_2, suggesting a high affinity of the protein to this lipid and the possible existence of a distinct PIP_2 binding site. A corresponding high affinity for PIP_2 was observed when the interaction of native band 3 with the pyrene-labelled derivative was measured. Moreover, lipid analyses on band 3 isolated in the presence of an excess of phospho-inositides suggest that as many as four PIP_2 molecules may bind to the protein. These analyses also indicate that PI is firmly associated with the protein. However, a high affinity of band 3 for pyrene-labelled PI was not observed, so that the relevance of the former finding is unclear. We conclude that the high affinity of band 3 towards PIP_2 and, to a lesser extent, towards PIP is an inherent property of band 3. From a comparison of the data in Table 1 and in [27] we infer that as much as 30% of the PIP_2 in the human erythrocyte membrane may be bound to band 3. This binding may well lead to segregation and different metabolic pools of PIP_2 in the intact erythrocyte membrane [12,24]. It remains to be determined whether these interactions have an effect on the functions of band 3.

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