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Red cell membrane sialoglycoprotein β in homozygous and heterozygous 4.1(−) hereditary elliptocytosis

N. Alloisio [a,*], L. Morlé [a], D. Bachir [b], D. Guetarni [b], P. Colonna [b] and J. Delaunay [a]

[a] *Groupe de Recherches sur le Globule Rouge, Faculté de Médecine Grange-Blanche, 69373 Lyon Cedex 08 (France) and* [b] *Centre Pierre et Marie Curie, Hôpital Mustapha, Alger (Algeria)*

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Sialoglycoprotein β, a minor sialoglycoprotein of the red cell membrane, was studied in homozygous and heterozygous 4.1(−) hereditary elliptocytosis, a variety of hereditary elliptocytosis characterized by total or partial absence of protein 4.1. Erythrocytes were treated with the periodic acid-NaB^3H_4 procedure. Following polyacrylamide gel electrophoresis in the presence of SDS, labelled sialoglycoproteins were revealed by fluorography. (i) In the ghosts from the 4.1(−) homozygote, sialoglycoprotein β was sharply decreased. It is not sure whether the residual material is sialoglycoprotein β itself, or a distinct sialoglycoprotein migrating in the same place. In long exposure fluorograms, sialoglycoprotein γ (a sialoglycoprotein related to sialoglycoprotein β) also turned out to be reduced. In the homozygote's Triton-shells, sialoglycoproteins β and γ appeared completely absent. (ii) In the 4.1(−) heterozygote, sialoglycoprotein β appeared slightly reduced, whereas sialoglycoprotein γ appeared normal. Both of these proteins were extracted in seemingly normal amounts in the Triton-shells. These observations bring further support to the view that there is an interaction between skeletal membrane protein 4.1 and sialoglycoprotein β, that is additional to other interactions between the former protein and the lipid bilayer and/or other transmembrane proteins.

Introduction

The red cell shape is controlled by a submembrane protein network, the membrane skeleton, composed of three major proteins, spectrin, actin and protein 4.1 (for review, see Refs. 1–3). This molecular assembly is attached to transmembrane protein band 3 through band 2.1, or ankyrin. Evidence has been presented recently for other attachment points between protein 4.1 and the lipid bilayer [4–6], glycophorin A [7] or any other, yet unidentified protein [8]. In addition, a minor sialoglycoprotein, termed PAS 2, glycophorin C [9] or sialoglycoprotein β [10], appears with skeletal proteins in the Triton-shells, a fact suggesting a linkage between this protein and the membrane skeleton [11]. In an elliptocytic child, Mueller and Morrison [12] have reported the contemporaneous absence of protein 4.1 and of this sialoglycoprotein, which they gave the name of glycoconnectin. However, Anstee et al. [13,14] described more recently individuals lacking sialoglycoprotein β (and sialoglycoprotein γ). Although some of them were mildly elliptocytic, all appeared to have normal amounts of protein 4.1.

* To whom correspondence and reprints requests should be addressed.

4.1(−) Hereditary elliptocytosis specifically refers to a variety of hereditary elliptocytosis associated with partial or total lack of protein 4.1 [15–18]. Its heterozygous state has been extensively characterized and has been termed the 4.1(−) trait [19]. In this work, we have investigated sialoglycoprotein β in a child with homozygous 4.1(−) hereditary elliptocytosis and her father, carrying the 4.1(−) trait. In the homozygote, sialoglycoprotein β was sharply reduced in the ghosts and did not appear at all in the Triton-shells. In the 4.1(−) trait carrier, sialoglycoprotein β was only slightly decreased in the ghosts and appeared normally in the Triton-shells.

Methods

The proposita, and Algerian girl, was born in 1972. Her parents are first cousins. She was initially referred to us for a severe hemolytic picture associated with elliptocytosis. Transfusion needs rapidly led to splenectomy, that was performed in 1975. In 1980, Tchernia et al. observed that she completely lacks protein 4.1 and that her parents display a partial reduction of this protein [15,16]. Presently, the proposita does not need blood transfusion. Upon recent examination, her red cell indices were as follows: red blood cells, 5.14 $T \cdot l^{-1}$; hemoglobin, 119 $g \cdot l^{-1}$; packed cell volume, 0.380; mean corpuscular volume, 74 fl. In the father, who is clinically normal, we found: red blood cells, 5.57 $T \cdot l^{-1}$; hemoglobin, 139 $g \cdot l^{-1}$; packed cell volume, 0.476; mean corpuscular volume, 85 fl. Informed consent was obtained from the patients and all procedures were performed according to the Declaration of Helsinki.

Blood was collected on citrate-citric acid dextrose medium and kept overnight at 4°C. Radioactive labelling of sialoglycoproteins was done essentially according to Mueller and Morrison [12]. Sialic acid residues of 1 ml washed red cells were oxidized with 3 volumes of a solution containing 5 mM sodium phosphate buffer (pH 7.40)/150 mM NaCl/1.33 mM sodium periodate. The reaction was carried out in ice for 10 min in the dark, and stopped with 2 volumes of 10 mM sodium arsenite in 5 mM sodium phosphate buffer (pH 7.40)/150 mM NaCl. Erythrocytes were centrifuged at 900 × g for 10 min. After three additional washes, red cells were labelled with NaB^3H_4 (1 mCi/ml of packed red cells) for 20 min at 25°C. Red cells were then washed again three times and ghosts were prepared according to Dodge et al. [20], in the presence of 0.1 mM phenylmethylsulfonyl fluoride.

Membrane skeletons were obtained by treating the ghosts with 8 volumes of cold 0.1% Triton X-100 (v/v) in a 5 mM sodium phosphate buffer (pH 7.40)/1 mM dithiothreitol [11]. The suspension was immediately mixed and centrifuged in an Eppendorf microcentrifuge for 6 min at 4°C. The pellet was washed once with the above Triton X-100 solution and subsequently with 5 mM sodium phosphate buffer (pH 7.40)/1 mM dithiothreitol. The first supernatant and the washed pellet fractions were immediately boiled in sample gel electrophoresis buffer and stored at −70°C.

Membrane proteins were analysed according to the procedure of Fairbanks et al. [21]. Sialoglycoproteins were separated on a linear 12–16% gradient SDS-polyacrylamide gel using the discontinuous system of Laemmli [22]. Proteins were stained by Coomassie blue R 250. The radioactivity was detected by fluorography at −70°C [23,24]. X-omat AR films Kodak were preexposed to a brief flash of light in order to obtain a fog absorbance of 0.15. By short and long exposure times, we will mean 5 and 35 days, respectively. Stained gels and fluorograms were scanned with a spectrodensitometer (Cellosystem Sebia), at 570 nm and without filter, respectively. The purpose of short exposure times was to keep proportional the fluorographic response (absorbance < 1.5) and the actual radioactivity of most of the bands. It alleviated the risk of underestimating heavily labelled bands and overestimating poorly labelled bands. In the ghosts, sialoglycoprotein β was tentatively quantified as the β/α and the $\beta/(\delta + \delta_2)$ ratios provided all bands concerned were strictly in the zone of proportionality. In the Triton-shells, there was no other sialoglycoprotein to serve as an internal standard.

Results

The Coomassie blue pattern of membrane ghost proteins appeared in good correspondence with those previously published [15,16]. In addition, we

observed that an extra-band appeared in the region of band 4.5, as we usually see in ghosts that cannot be completely freed of hemoglobin (Fig. 1). This band was also present among Triton-soluble proteins. Finally, band 4.9, recently identified as an actin-bundling protein [25], seemed to be duplicated. In the 4.1(−) trait carrier, the band 4.1 percentage (with respect to band 3) was 13.1%, a value in strict agreement with that found in seven independent 4.1(−) trait carriers (12.5% ± 0.7% vs. 17.7% ± 1.2% in 14 controls) [19]. None of these other abnormalities was recorded in the heterozygote.

The fluorographic patterns are shown in Figs. 2 and 3. In ghosts, sialoglycoprotein β (36.5 kDa) was clearly visible provided electrophoresis lasted long enough so as to separate the band β from the band α. In short exposure fluorograms, $(\delta + \delta_2)/(\alpha + \alpha_2)$ ratios ($\times 100$) were comparable for control, proposita and the 4.1(−) trait carrier (16.3, 19.3 and 24.3, respectively) and in a similar range as reported by Furthmayr [9]. High molecular weight material remaining on top of the gel was in all cases less than 6%. Sialoglycoprotein β was quantitated using the sum of densities between band $\alpha\delta$ and δ as a basis, and omitting band α_2 because this heavy band was out of the densitometric proportionality ($E < 2.2$–2.4).

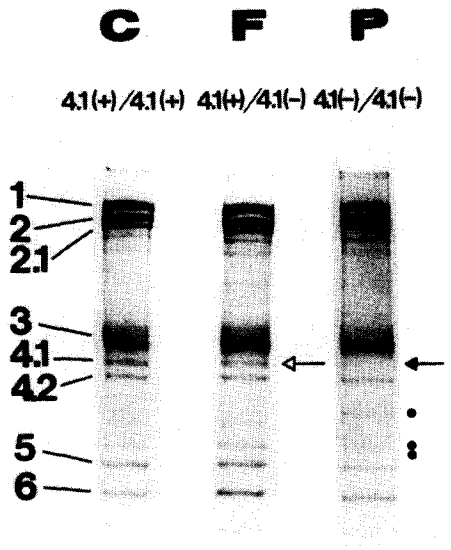

Fig. 1. Electrophoretic pattern of Coomassie blue-stained proteins. SDS-PAGE was carried out according to Fairbanks et al. [21]. C, controls; F, father (with the 4.1(−) trait); P, proposita (with homozygous 4.1(−) hereditary elliptocytosis). Bands were numbered after Fairbanks et al. [21]; ◁—, 30% decrease of protein 4.1 as detected by scanning (not shown) (this reduction is regularly observed in the 4.1(−) trait [19]); ◀—, total absence of protein 4.1; •, the extra-band in the region of protein 4.5; :, the band 4.9 duplication. Scanning failed to detect any band 4.1 in the proposita (not shown).

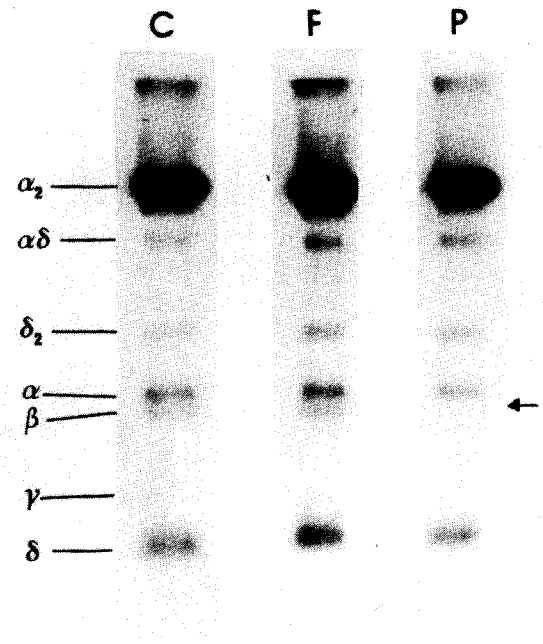

Fig. 2. Fluorographic autoradiograms after a short exposure time. C, control; F, father; P, proposita. Sialoglycoproteins were analyzed on SDS-PAGE according to Laemmli, using a linear 12–16% acrylamide monomer gradient [22]. Bands and peaks are lettered after Anstee et al. [10]. Band α corresponds to the glycophorin monomer, and can form a major homodimer α_2. Sialoglycoprotein β appears separated from band α. ◀—, the sharply reduced sialoglycoprotein β in P. On this short exposure fluorogram, sialoglycoprotein γ is nearly undetectable. The molecular weight markers are 84000, 44500, 36500 and 26500 for bands α_2, δ_2, β and δ, respectively.

In homozygous 4.1(−) hereditary elliptocytosis, sialoglycoprotein β from the ghosts appeared sharply reduced (Table I, Fig. 2). The long exposure fluorogram showed that sialoglycoprotein γ is reduced and that the faint band X_1 (33.5 kDa) of the control is absent. In contrast, the faint band X_2 (32.5 kDa) appears or at least becomes much

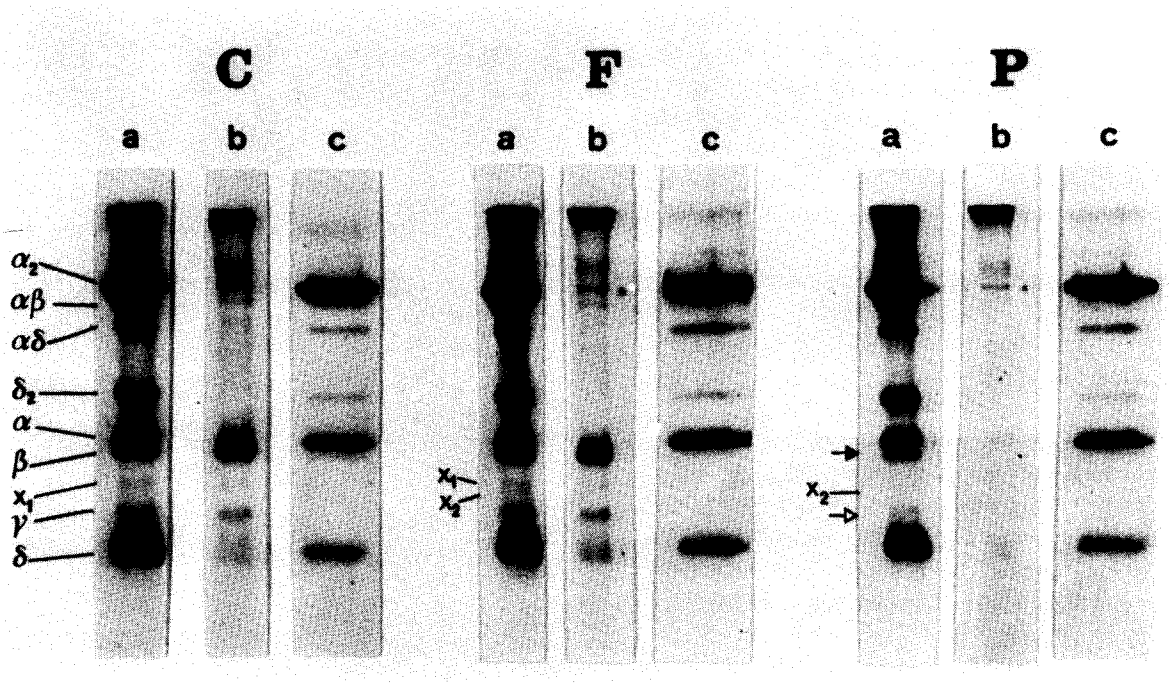

Fig. 3. Fluorographic autoradiograms after a long exposure time of ^{3}H-labelled sialoglycoproteins from the ghosts (a), the Triton-shells (b), and the Triton-soluble fraction (c). The $(\delta + \delta_2)/(\alpha + \alpha_2)$ ratio ($\times 100$) was between 40 and 50, indicating that highly labelled bands fell out of the zone of proportionality. The percentages of radioactivity on top of the gel was approx. 12%. C, control; F, father; P, proposita. Bands are lettered after Anstee et al. [10]. Sialoglycoprotein γ (30 kDa) is well visible in the control and the 4.1(−) trait carrier. In P, sialoglycoprotein β (—►) was sharply reduced in the ghosts and completely absent from the Triton-shells; sialoglycoprotein γ (—▷) was also reduced in the ghosts and absent from the Triton-shells. X_1 and X_2 are minor sialoglycoproteins. X_1 must correspond to normal band β_1 described by Anstee et al. [13,14]. It is present in the control and the 4.1(−) trait carrier. X_2 is an abnormal component, existing only in homozygous or heterozygous 4.1(−) hereditary elliptocytosis.

TABLE I

THE ESTIMATION OF SIALOGLYCOPROTEIN β AND OTHER SIALOGLYCOPROTEINS, AFTER A SHORT EXPOSURE TIME

The scanning covered the gel from band $\alpha\delta$ to band δ (the band α_2 was omitted due to its high absorbance, see text). The amount of each is expressed as the ratio ($\times 100$) of its absorbance to the overall absorbance (within the scanning range). In brackets: the decrease, tentatively expressed as percentage of the β/α and $\beta/(\delta + \delta_2)$ ratios in heterozygous and homozygous 4.1(−) hereditary elliptocytosis with respect to control. These figures correspond to one gel in which all bands from $\alpha\delta$ to δ were strictly in the zone of proportionality.

	Control 4.1(+)/4.1(+)	Father 4.1(+)/4.1(−)	propositus 4.1(−)/4.1(−)
δ	38.9	36.7	34.7
β	7.6	6.4	2.3
α	20.2	18.2	18.2
$\alpha\delta$	10.8	15.4	16.0
δ_2	9.4 [a]	12.2 [a]	15.6 [a]
$\beta/\alpha \times 100$	37.6	35.2 (−6.4%)	12.6(−66.5%)
$\beta/(\delta + \delta_2) \times 100$	15.7	13.1(−16.6%)	4.6(−70.7%)

[a] The sum $\delta + \delta_2$ is constant in all cases.

more prominent (Fig. 3). Most of the bands being out of densitometric proportionality, no quantitation of bands γ, X_1 and X_2 was possible. In the Triton-shells, long exposure fluorograms showed that sialoglycoproteins β, X_1 and γ were absent, in sharp contrast with their presence in the control (Fig. 3). Band X_2 was also absent from the Triton-shells.

In heterozygous 4.1(−) hereditary elliptocytosis, sialoglycoprotein β appeared slightly reduced in the ghosts (Table I, Fig. 2). Upon long exposure times, sialoglycoprotein γ was present in seemingly normal amounts and both bands X_1 and X_2 were noted. In the Triton-shells, sialoglycoproteins β and γ also appeared present in normal amounts (however, as stated above, the absence of any band serving as in internal standard made it impossible to quantitate these bands, even when short exposure fluorograms were carried out (not shown)).

Discussion

Several works have suggested that protein 4.1 interacts with the lipid bilayer [4–6], or specific transmembrane proteins such as glycophorin A [7] or any other, yet unidentified protein [8]. The hypothesis of a specific interaction of protein 4.1 with sialoglycoprotein β arose from the contemporaneous absence of sialoglycoprotein β and protein 4.1 in an elliptocytic child [12]. However, since no genetic information was provided, the cause of the absence of protein 4.1 remains uncertain. In this work, we present additional data on this question, based on the study of homozygous and heterozygous 4.1(−) hereditary elliptocytosis.

In homozygous 4.1(−) hereditary elliptocytosis, the reduction of sialoglycoprotein β was clearly visible. Since the amount of material remaining on top of the gel was less than 6% of total material and since it was not increased in the proposita, it is unlikely that the missing sialoglycoprotein β has been retained at this level. The possibility exists that the material still present in the place of sialoglycoprotein β is a distinct sialoglycoprotein. The reduction of sialoglycoprotein γ in the ghosts and its absence from the Triton-shells in homozygous 4.1(−) hereditary elliptocytosis, reproducing the behaviour of sialoglycoprotein β, brings additional support to the view that it is related to the latter. Band X_1 also conforms essentially to this behaviour. In contrast, band X_2, which appears in the homozygote or the heterozygote, and fails to be extracted in the Triton-shells, seems to belong to a distinct category. In the persons reported by Anstee et al. [13,14], two of whom only present an elliptocytosis, band β, β_1 and γ are also missing. Band β_1, to which band X_1 of this work may correspond, is sometimes replaced by diffusely migrating components [14]. Band ε has the same apparent molecular weight as band X_2 of this work.

The reduction of sialoglycoprotein β in the ghosts from the present 4.1(−) trait carrier appears much more limited than in the homozygote. In a separate family with two additional 4.1(−) trait carriers, we found that sialoglycoprotein β was also reduced, although to higher extents [26]. These fluctuations probably result, at least in part, from the difficulty of fluorographic measurements, even though we carefully operated in the zone of densitometric proportionality. The variations of the $\alpha \rightleftharpoons \alpha_2$ and $\delta \rightleftharpoons \delta_2$ equilibria from one preparation to another, those of the red cell age distribution, and the Ss phenotype (for δ quantitation) may also become more critical. In the 4.1(−) trait, further studies will therefore be necessary to evaluate unambiguously the amount of sialoglycoprotein β.

Although no in vitro binding test has yet been feasible, it seems reasonable to hypothesize that the defects of protein 4.1 and sialoglycoprotein β are causally related. It has been presented that a single red cell contains approximately 200 000 and 35 000 molecules of protein 4.1 and sialoglycoprotein β, respectively [1,27]. As a result, the partial lack of protein 4.1 may have sensible, albeit variable, effects on the amount of sialoglycoprotein β as is exemplified in the 4.1(−) trait. Possibly, protein 4.1 stabilizes sialoglycoproteins β, X_1 and γ in the lipid bilayer.

We postulate that the primary defect of 4.1(−) hereditary elliptocytosis involves protein 4.1 and that the abnormalities of sialoglycoprotein β, X_1 and γ are secondary. The nature of the 4.1(−) gene may be questioned. In this 4.1(−) trait carrier as well as in other carriers [19,26], the constant absence of any additional band in the region of band 4.5 does not support the view that this band

would be a degradation product of protein 4.1. In two 4.1(−) carriers, anti-4.1 monoclonal antibodies failed to reveal any band in this region [26]. We have suggested that the 4.1(−) gene be an allele of the thalessemic type [26].

Among the persons reported by Anstee et al., who lacked sialoglycoproteins β, β_1 and γ, only two display a mild elliptocytosis, while the others are morphologically normal [13,14]. It may appear conflicting that the persons who are elliptocytic have an apparently normal amount of protein 4.1, unless one considers that only the mode of attachment of protein 4.1, and not its amount, is affected. From studies on the 4.1(−) trait, we know that the absence of one haploid set of protein 4.1 invariably generates a 100% elliptocytosis and that the electrophoretic recognition of this absence requires high accuracy densitometric measurements [19]. A more limited decrease of protein 4.1 may, therefore, be overlooked. In the conditions described by Anstee et al. [13,14], the primary defect could involve sialoglycoproteins β, β_1 and γ, resulting in a small reduction of protein 4.1, that would hardly be detectable electrophoretically, although it would be sufficient to generate a mild elliptocytosis in some cases. It would be interesting to assay the Gerbich antigen in 4.1(−) hereditary elliptocytosis, since this antigen is absent in the cases described by Anstee et al. [13,14].

The present work provides further evidence for the existence of a protein 4.1-sialoglycoprotein β nexus. Because protein 4.1 largely saturates sialoglycoprotein β, this nexus would not have the same function as the interaction between spectrin, ankyrin and band 3, in which band 3 stoichiometrically predominates. It would further complicate the interaction of protein 4.1 with the lipid bilayer [4–6], glycophorin A [7], or any other protein [8].

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