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Alterations of Globin Chain Synthesis and of Red Cell Membrane Proteins in Congenital Dyserythropoietic Anemia I and II

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Red cell membrane proteins were investigated in two unrelated children with congenital dyserythropoietic anemia (CDA) I and two siblings with CDA II. The CDA I patients displayed globin chain synthesis imbalance, with reduction of the non α/α ratio. One of the CDA II patients presented the reverse alteration. Whenever globin chain synthesis was unbalanced, the membrane *p*-nitrophenylphosphatase had an abnormally biphasic kinetics, consistent with substrate excess inhibition, as is observed in α- or β-thalassemic syndromes. One CDA I patient displayed a decrease of electrophoretic band 4.1 along with an ectopic phosphorylated protein at the level of band 4.2. In CDA II patients, band 3 was strikingly narrower than in controls. In CDA II and, to a lesser extent, in CDA I, the *in vitro* endogenous phosphorylation of band 2 + 2.1 was sharply reduced.

Congenital dyserythropoietic anemia (CDA) is a category of rare refractory anemias, inherited as autosomal recessive traits and characterized by ineffective erythropoiesis. Remarkable morphologic alterations have been observed both in the bone marrow and in the blood, setting the basis for the current classification of these disorders. Among the most common findings are multinuclearity, karyorrhexis, and basophilic stippling. More specifically, one finds abnormal chromatin structure including internuclear bridges in CDA I (26) and the presence of an additional membrane structure next to the plasma membrane in CDA II (41, 45). Furthermore, CDA II erythrocytes are unusually lysed by an alloantibody (anti-HEMPAS) present in many normal sera (13) and agglutinated by anti-i.

In contrast to CDA I membranes, a great variety of abnormalities have been described in CDA II membranes: a decrease of the neuraminic acid content (6, 22), an increase of lecithin and glycolipids (30, 31), a decrease of ethanolamine phosphoglycerides (38), changes of the number and/or the intensity of minor protein bands (10, 22) and a reduction of band 3 (4, 5, 18, 24).

Previous works have shown that CDA could display some features of thalassemic syndromes, including globin chain synthesis imbalance (18, 28, 42) and increased sensitivity of erythrocytes to H_2O_2 treatment (38). In the present study, we investigated globin synthesis and red cell membrane proteins in two unrelated CDA I and two related CDA II children. Because the membrane neutral phosphatase displays typical kinetic alteration in thalassemia (36, 37), we also studied this enzyme in the CDA patients and found similar alterations provided globin chain synthesis was unbalanced. In addition, we describe a decrease of membrane protein endogenous phosphorylation, a nonspecific feature occurring in a variety of hemolytic anemias. Finally, we observed some more specific changes, such as a reduction of band 3 in CDA II patients.

PATIENTS AND CONTROLS

Patients 1 and 2 display CDA I and patients 3 and 4 CDA II. Patient 1 (female) was born in 1965 from nonconsanguineous parents originating from the southern part of France. Her parents and brother are clinically and hematologically normal. Detailed case report has been published elsewhere (29). Patient 2 (male) was born in 1978 from nonconsanguineous parents originating from the Reunion Island. He carries the sickle cell trait, which he inherited from his mother. Patients 3 and 4 are two sisters born in 1967 and 1970, respectively, from nonconsanguineous parents originating from the Jura mountains. The parents and four other children are apparently normal, as far as we know. Another child of the fratry also suffers, presumably, from CDA II, but was not included in this study due to limiting routine laboratory data.

Table 1. *Routine laboratory data*[1]

	CDA I		CDA II	
	1	2	3	4
Erythrocytes ($10^{12} \cdot l^{-1}$)	3.09	3.57	3.71	3.88
Hb ($g \cdot dl^{-1}$)	9.4	9.1	12.3	11.6
PVC (%)	27.7	27.4	34.5	33.4
MCV (fl)	86.0	76.0	93.0	86.0
MCH (pg)	30.5	25.2	32.9	29.6
MCHC ($g \cdot dl^{-1}$)	33.9	32.8	34.3	33.3
Abnormal shapes	+	+	−	+
Reticulocytes (%)	2.6	3.0	4.0	3.0
Serum iron ($\mu mole \cdot l^{-1}$)	50.0	14.5	39.4	43.1
Acidified serum test	−	−	+	+
Anti-i	normal	normal	1/1280	1/5120
Basophilic stippling	+	+	+	+
Multinucleated erythroblasts/number of late erythroblasts (%)	15	8	20	n.d.
Karyorrhexis	+	+	+	+
Double membrane	−	n.d.	+	+

[1] White blood cells and platelets were normal. Erythrokinetic patterns (patients 1, 2 and 4) showed ineffective erythropoiesis. Abnormal shapes refer to the presence of poikilocytes, anisocytes, schizocytes and/or elliptocytes. n.d., not determined and CDA, congenital dyserythropoietic anemia.

Clinically, the children display an usually severe anemia with various short and long term complications. Routine laboratory and morphologic data are presented in Table 1 and Figure 1, respectively. Acidified serum test was found negative in CDA I. It was found positive in CDA II patients provided fresh neutral sera were used. Controls were healthy volunteers.

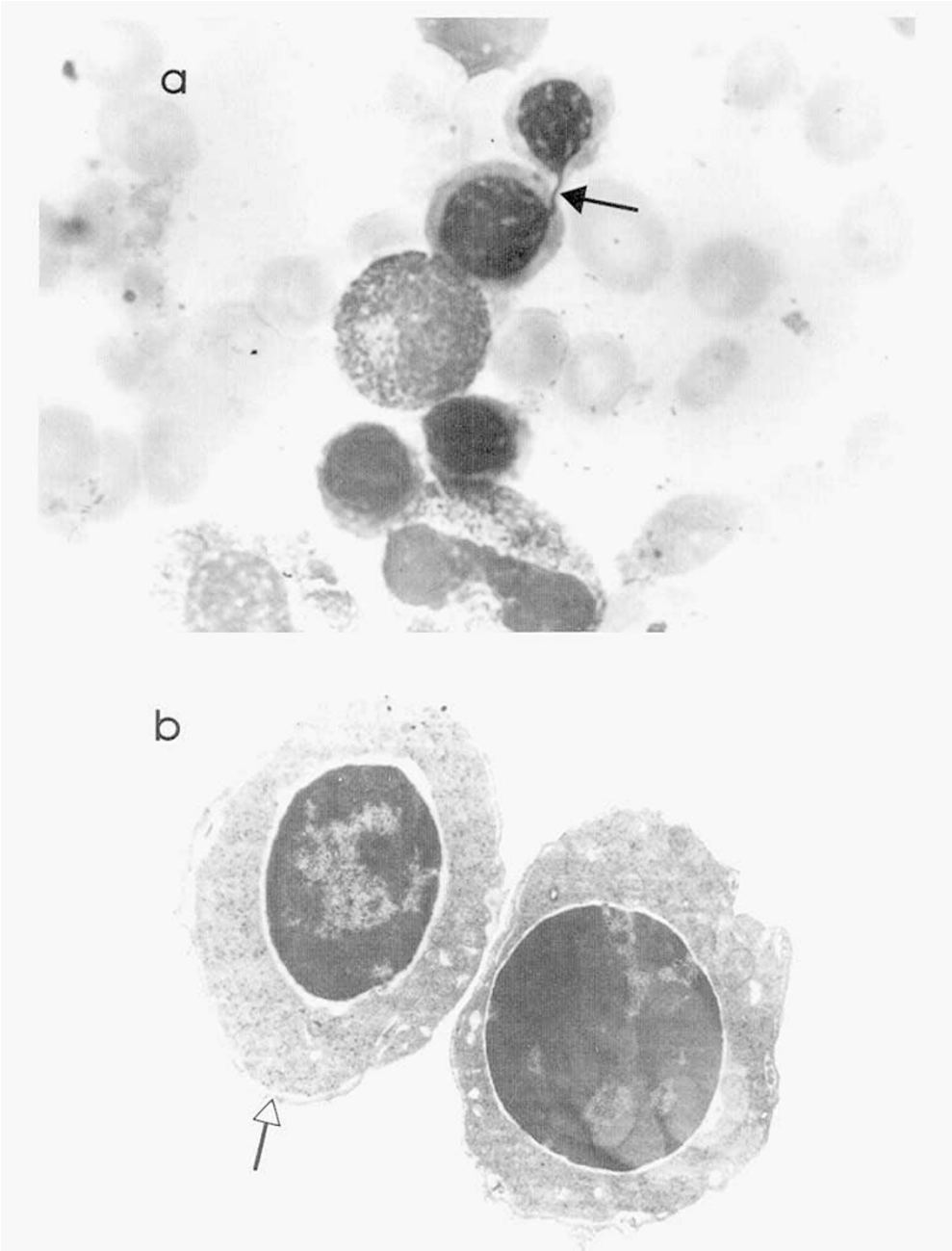

Fig. 1. Morphologic data. (*a*) Internuclear chromatin bridges (➞)connecting two erythroblasts in patient 2 and characteristic of CDA I and (*b*) electron micrograph of a binucleated erythroblast with partial double membrane (➞▷) in patient 4 and characteristic of CDA II.

MATERIALS AND METHODS

The origin of the compounds is as follows: acrylamide, bisacrylamide, Serva; TEMED, Baker; cAMP and pNPP, Sigma Chemical Co.; and [γ-^{32}P]-ATP, The Radiochemical Center. Most of other organic and mineral compounds were obtained from Merck.

In vitro globin chain synthesis. In vitro globin chain synthesis was carried on essentially according to Lingrel and Borsook (33) using L-[4,5-^{3}H]-leucine as a tracer. Globin chains were separated by ion-exchange chromatography on DE 52 cellulose (12). Synthesis imbalance was expressed as the ratio of total radioactivity incorporated into non-α (β and/or γ) chains to α chains (non α/α ratio).

Preparation of erythrocyte ghosts. Blood was collected on citric acid dextrose medium. Erythrocyte ghosts were prepared essentially according to Dodge *et al.* (16), with some modifications (14) and stored at −70°C. For enzymatic studies, they were thawed only once. Before analysis, they were washed twice with 5 mM Tris buffer solution (pH 7.5).

p-Nitrophenylphosphatase (pNPP) assay. *p*-Nitrophenylphosphatase was analysed as described elsewhere (15, 36, 37). Specific activity (SA) and maximum velocity (V_{max}) were expressed as nmoles pNPP hydrolyzed per mg protein per min (nmol·mg^{-1}·min^{-1}). Double reciprocal plots (SA^{-1} *versus* [pNPP]$^{-1}$) allowed to determine V_{max} and K_{Mapp} in the case of Michaelis-Menten kinetics and in the ascending part of the curve ([pNPP] < 10 mM) when biphasic kinetics existed. In order to quantify the inhibition by substrate excess, reflected by the descending part of the curve, we determined the 25/2.5 ratio, *e.g.*, the ratio of SA at 25 mM (nearly saturating concentration of substrate under normal conditions) to that at 2.5 mM (approximately the value of K_{Mapp}).

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to a technique derived from that of Fairbanks *et al.* (19). Proteins were assayed by the Lowry procedure (34). They were preincubated (1 mg/ml) at 60°C for 15 min in the following medium: 1% SDS, 0.5 mM EDTA and 6 mM β-mercaptoethanol (1, 2). Fifteen μl aliquots were submitted to electrophoresis in a gel slab (8.2 x

8.2 x 0.27 mm; 5.6% acrylamide and 0.21% bisacrylamide monomers), in a medium (pH 7.4) containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA and 0.2% SDS. The conditions of electrophoresis were: 70 V with a current of 50 mA per gel until the bromophenol tracking dye reached the bottom of the gel. Staining with Coomassie blue was performed using standard procedures.

Membrane protein in vitro phosphorylation. Phosphorylation of membrane proteins was carried out according to a technique derived from that of Roses and Apple (40). Two hundred μg of proteins were incubated at 37°C for 5 min in 200 μl of a medium containing 50 mM sodium acetate (pH 6.0), 10 mM magnesium acetate, 2.5 μM [γ-^{32}P] ATP (SA, 2–4 Ci·mole^{-1}) and, in some cases, 5 μM cAMP. After 5 min, the reaction was stopped with 20 μl of a solution containing 10% SDS, 5 mM EDTA and 60 mM β-mercaptoethanol. The mixture was submitted to SDS-PAGE. For determination of the phosphorylation of individual proteins, parts of the gel containing the major protein bands were sliced off, dissolved, and counted. Phosphorylation of individual proteins was expressed as pmoles of Pi incorporated per mg of applied proteins per 5 min (pmole/mg/5 min). Alternatively, polyacrylamide gel slabs were dessicated and revealed by autoradiography (Ultrofilm ^{3}H LKB 2208-190).

RESULTS

Globin chain synthesis ratio (Table 2). The non α/α ratio was significantly decreased ($<m - 2\sigma$) in patients 1 and 2 and significantly increased ($>m + 2\sigma$) in patient 4. It was normal in patient 3. The % of HbA_2 in patient 1 and his parents, and the normal non α/α ratio in the latter allowed us to rule out the possibility of an accompanying β-thalassemic trait in this family. In patient 2, the presence of HbS would decrease the non α/α ratio but never to such a low value. In this patient and his relatives, the existence of HbS accounts by itself for the slightly elevated HbA_2 (21, 44). In patient 4 we cannot ascertain the absence of an α-thalassemic trait (α-thalassemia-2), although the ethnic background makes it extremely unlikely.

Abnormal p-nitrophenylphosphatase kinetics (Table 3, Fig. 2). Except for patient 3, the *p*-nitrophenylphosphatase displayed an abnormally biphasic character, consistent with substrate excess inhibition (36, 37). This change is similar to that encountered in the thalassemic syndromes although, in CDA, we failed to observe a decrease of K_{Mapp}. In addition, the 25/2.5 ratio was lower in CDA than in α- or β-thalassemic syndromes with comparable non α/α ratios. It is noteworthy that patient 3 had normal kinetics along with balanced globin chain synthesis.

Table 2. *Globin chain synthesis ratio and related data*[1]

	HbA_2 (%)	HbF (%)	Nonratio
CDA I			
1	1.04	3.60	0.77
F	1.37	2.65	1.12
M	1.37	2.65	1.05
B	1.23	1.50	0.99
2°[2]	3.68	7.13	0.76
M°°[2]	3.24	0.62	n.d.
B°°°[2]	3.23	1.07	n.d.
CDA II			
3	2.62	2.50	1.03
4	2.13	1.50	1.55
Controls	3.5	2	1.06 ± 0.09 (n = 10)

[1] Hb A_2 and Hb F were determined by microchromatofocusing (20) and the use of alkali (7), respectively. In some cases, relatives of the propositus were examined: father (F), mother (M), brother (B). For patients, the non α/α ratios are the mean of two or three tests with independent blood samples. Underlined figures indicate those values of the non α/α ratio that are outside the m ± 2σ interval. n.d., non determined.

[2] °HbS = 33.7%; °°HbS = 34.4%; °°°HbS = 34.5%. Normal values, 35–36%, using microchromatofocusing (20).

Membrane protein electrophoretic profiles (Fig. 3). In CDA I patient 1, the profile was normal. In CDA I patient 2, the intensity of band 4.1 was reduced, a feature which we will consider along with the existence of an ectopic phosphorylated protein at the level of band 4.2 (see below). Also in patient 2, extra-proteins appeared at the level of bands 2.4 and between bands 5 and 6. In the two CDA II patients, band 3 appeared clearly narrower than in controls, a highly unusual aspect previously described by other authors (4, 5, 18, 24).

Membrane protein endogenous phosphorylation (Fig. 4). Decrease of major phosphorylated bands 2 + 2.1 and 3 was apparent on radioautograms, both in the absence and in the presence of cAMP. Gel slice counting showed that the reduction was most conspicuous for bands 2 + 2.1 and was usually more pronounced in CDA II patients than in CDA I patients. In CDA I patient 2, an ectopic phosphorylated band was observed at the level of protein 4.2 in the presence of cAMP, a fact to be brought together with the above mentioned decreased intensity of Coomassie blue band 4.1. Still in CDA I patient 2, additional phosphorylated bands also appeared at the level of band 2.4 and between bands 5 and 6. In all patients, the phosphorylation of other bands, as determined by radioautography and gel slice counting, was normal or not significantly altered (*e.g.*, within the m ± 2 σ interval) (not shown).

DISCUSSION

To our knowledge, no disturbed globin chain synthesis has been described in CDA I (25) whereas imbalance leading to a decrease

Table 3. *p-Nitrophenylphosphatase parameters*[1]

Patients	V_{max} (nmole/mg/min)	K_{Mapp} (mM)	25/2.5 ratio
CDA I			
1	4.49	2.58	1.42
2	3.84	2.25	1.17
CDA II			
3	8.66	4.32	1.84[2]
4	8.86	2.34	1.40
Controls	2.72 ± 0.67 (n = 20)	2.78 ± 0.72 (n = 22)	1.98 ± 0.16 (n = 20)

[1] Except for patient 3, the kinetics was biphasic (Fig. 1).

[2] Nonbiphasic kinetics.

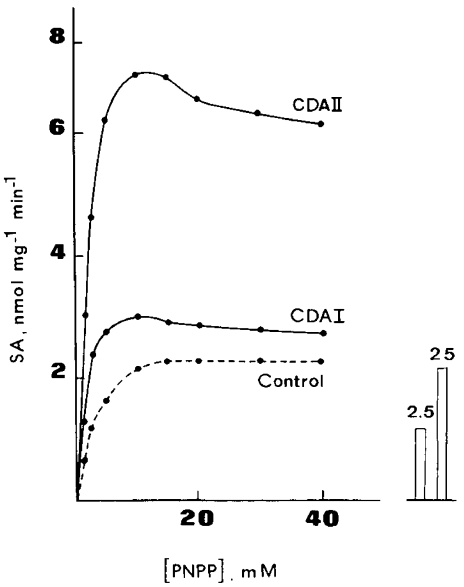

Fig. 2. *p*-Nitrophenylphosphatase kinetics. In CDA I and II, the pNPP concentration curve was biphasic, in contrast to controls in whom it was of the Michaelis-Menten type. The way the 25/2.5 ratio was calculated is visualized for controls. CDA I, patient 2 and CDA II, patient 4.

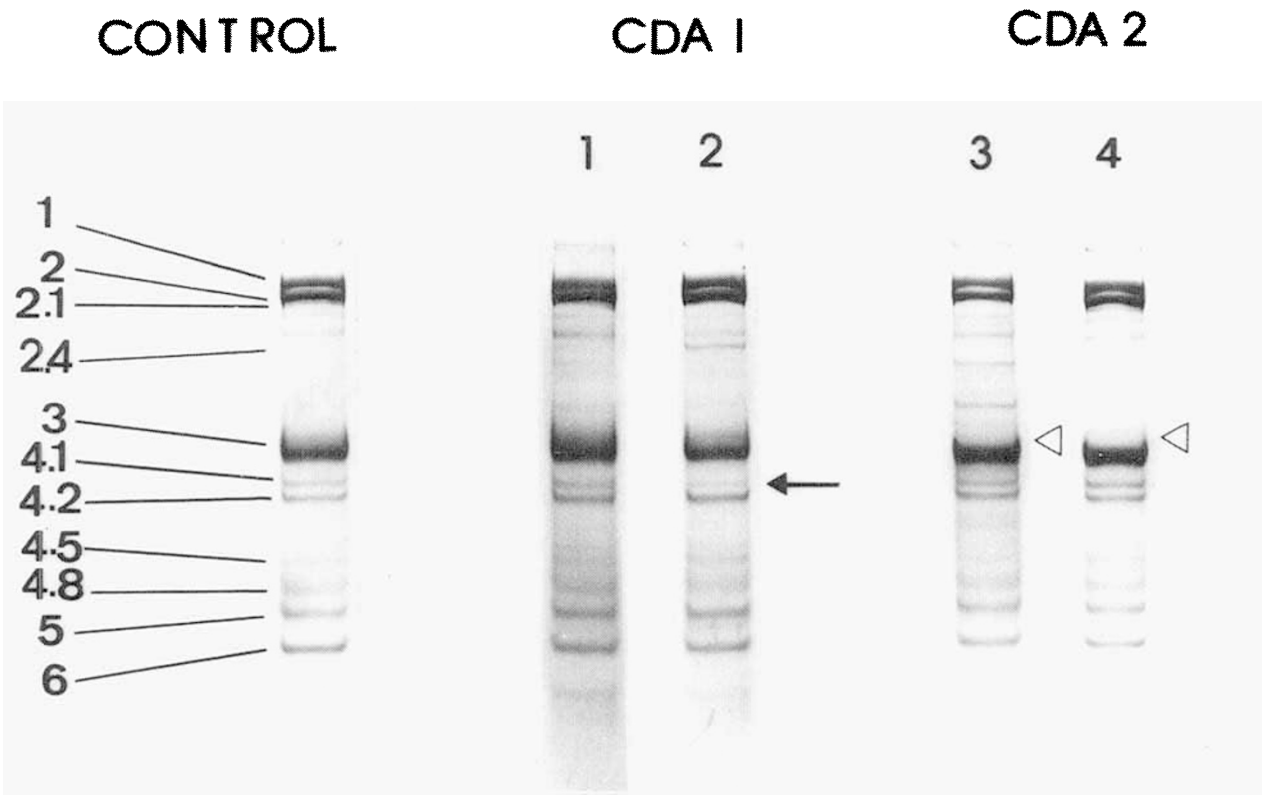

Fig. 3. Membrane protein electrolphoretic patterns. SDS-PAGE was carried out as described in the text. Fifteen μg protein per sample were applied. Major Coomassie blue bands were numbered according to Fairbanks *et al.* (19). Bands 1 and 2 correspond to the α and the β chains of spectrin, respectively, a major cytoskeletal protein. Broad band 3 contains, among other proteins, the anion channel protein. Bands 4.1 and 5 (actin) are the two other major cytoskeletal proteins. (➞), band 4.1 with drastically reduced intensity in CDA I patient 2 and (▷), narrower band 3 in the CDA II patients. Spectrin proteolysis was apparent in patient 3 (extra bands in the band 2-band 3 interval).

of the non α/α ratio has been reported in CDA II (18, 28). Weatherall *et al.* (42) also reported a decrease of the non α/α ratio in a familial case that he did not firmly identify to any type of CDA. Therefore, our observation of unbalanced synthesis with reduced non α/α ratio in CDA I and increased non α/α ratio in CDA II is at variance with the data available in the literature. Increased non α/α ratio has been recorded in sideroblastic anaemia (43). Although we did not study globin chain synthesis in bone marrow cells, it has been observed by other authors in various cases of CDA that unbalanced synthesis existed in precursor cells whenever it was recorded in peripheral blood (18, 25, 28, 42).

The factors governing globin chain imbalance must be highly variable from one patient to another. Variations must even exist in the same fratry, as is exemplified by the different non α/α ratios encountered in patients 3 and 4. To our knowledge, CDA I patient 2 is the first reported case combining a CDA (of any type) and a structural variant of hemoglobin (that turns out to serve as a useful marker). Unlike patients associating β-thalassemia and sickle cell traits, the HbS % was not increased. Nor was the β^S/β^A ratio unusual after *in vitro* globin chain synthesis ($\beta^S/\beta^A = 0.70$, a value underestimated due to a "pre β^S" peak cochromatographying with the β^A peak). These data support the view (42) that decreased non α/α ratio derives from an overproduction of α chains rather than from a defective β chain synthesis. If β chain synthesis was really defective, at least would the defect concern equally the production of β^A and β^S chain, the genes of which are in *trans* to each other. This would imply a mechanism entirely different from that existing in β-thalassemias.

The structural basis of the phosphatase biphasic kinetics is unknown at the present time. It appears nearly always associated with globin chain synthesis imbalance and the biphasic character increases in proportion as the imbalance becomes more pronounced (36, 37). The biphasic kinetics was constantly absent in other forms of hemolytic anemias investigated to date: sickle cell disease (15), hereditary spherocytosis or elliptocytosis (unpublished), hemoglobinopathies with severe hemoglobin unstability (37). One intriguing exception, however, concerns a familial case of hereditary stomatocytosis (11, 15) devoid of thalassemic symptom, in whom, however, *in vitro* globin chain synthesis could not be carried out. The presence of biphasic kinetics in those CDA I and CDA II patients who display an imbalance in globin chain synthesis strengthens the view that the former is related to the latter. One possibility is that the relationship is mediated by some oxidative damage of the membrane. O'Regan *et al.* (38) reported vitamin E deficiency in a patient with CDA II and assigned this deficiency to the utilization of the vitamin in stabilizing cellular membranes.

Decreased width of band 3 observed in our CDA II patients entirely agrees with previous observations (4, 5, 18, 24). The association in CDA I patient 2 of a reduced intensity of protein 4.1 and an ectopic phosphorylated band at the level of protein 4.2 realizes a highly unusual pattern, which we recently recognized (1). We hypothesized (1) and then confirmed (2) that this association was controlled by a defective allele, encoding a shortened variant of protein 4.1 that remains morphologically silent at the hererozygous state (2). But in CDA I patient 2, the presence of additional Coomassie and phosphorylated bands complicates the picture (Fig. 3 and 4). Therefore, further experiments and family study should clarify this question.

Decreased endogenous phosphorylation of membrane proteins was especially remarkable for bands 2 + 2.1, and to a lesser extent, in band 3 in CDA II patients. Although bands 2 and 2.1 represent distinct proteins, spectrin-component 2 and ankyrin, respectively, it was not possible to slice them separately because they are practically in contact. Decreased labeling did not result from membrane protein underestimation due to globin contamination because the latter did not appear in electrophoretic profiles (Fig.

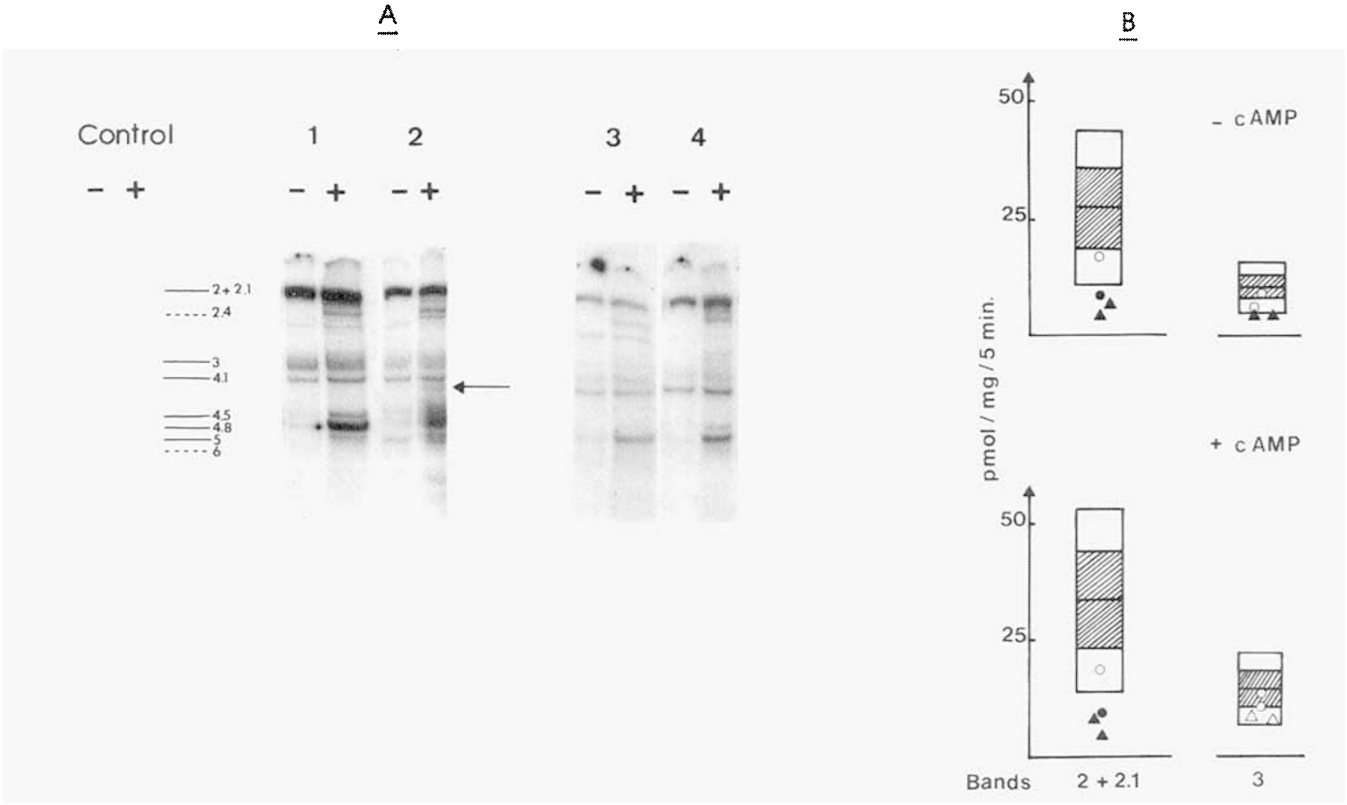

Fig. 4. Membrane protein endogenous phosphorylation patterns. (*A*) Radioautograms. Major phosphorylated bands were numbered according to Fairbanks *et al.* (19). (−), no cAMP and (+), 5 μM cAMP. Bands 2 and 2.1 phosphorylation was decreased in most patients. (➞), presence of an ectopic phosphorylated band at the level of protein 4.2 in the presence of cAMP (patient 2). In patient 3, extraphosphorylated bands in the band 2-band 3 interval are the expression of spectrin artefactual proteolysis. (*B*) Quantitative determination of radioactivity after gel slicing in the absence of cAMP (top) and in the presence of 5 μM cAMP (bottom). Values are expressed as pmole phosphate incorporated per mg of applied membrane proteins per 5 min. Only data that pertain to gel slices containing bands 2 + 2.1 or band 3 are shown. (°), CDA I patients 1 and 2. (△), CDA II patients 3 and 4. Closed marks refer to values outside the m ± 2σ intervals. The *hatched* and *open rectangles* represent the m + σ and the m ± 2σ intervals, respectively, for controls (n = 13). Bands 2 and 2.1 m ± σ = 27.1 ± 8.4 and 34.1 ± 10.2 without and with cAMP, respectively. Band 3: m ± σ = 10 ± 2.6 and 14.7 ± 1.1., without and with cAMP, respectively.

3). Reduced endogenous phosphorylation of bands 2 + 2.1 has been described in a variety of unrelated conditions, including homozygous sickle cell disease (8, 17, 27), hereditary stomatocytosis (9, 35), and hereditary elliptocytosis (1). We therefore assume that it reflects many different kinds of cytoskeletal protein rearrangements. Decreased phosphorylation of band 3 in CDA II was not necessarily the simple consequence of the narrowing of this band. At this stage, we cannot rule out that protein-kinase activities were also reduced.

In this study, we have described a variety of biochemical alterations concerning globin chain synthesis and membrane proteins in several cases of CDA I and CDA II. Abnormalities such as globin chain synthesis imbalance and phosphatase biphasic kinetics seem to partly relate CDAs to thalassemias. We assume that phosphatase alteration derives in some way from globin chain synthesis imbalance. Other abnormalities appear to be nonspecific, as is the case of band 2 + 2.1 reduced endogenous phosphorylation. In contrast, the narrowing of band 3 is probably highly specific of CDA II. At the present time, it is not possible to crystallize both these data and the well established immunologic and morphologic changes into a simple picture. Further work will be needed in order to understand how a probably single mutation can produce such pleiotropic effects.

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