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Phosphorylation of Component a of the Human Erythrocyte Membrane in Myotonic Muscular Dystrophy

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Summary. Endogenous membrane protein kinase activity in fresh erythrocyte ghosts is altered in myotonic muscular dystrophy. Phosphorylation of erythrocyte Component a, which migrates with an apparent molecular weight of 90,000 to 100,000, is significantly reduced compared to age- and sex-matched controls. The difference in endogenous membrane protein kinase activity in fresh RBC membranes lends confirmation to the suggestion that myotonic dystrophy is a disease of widespread membrane alterations.

Previous studies from our laboratory have demonstrated a reduction in the endogenous protein kinase activity in the red blood cell ghosts derived from patients with myotonic dystrophy [22]. Since this demonstration required freezing at -20 °C for one week it was difficult to delineate the factors responsible for the altered phosphorylation other than implicating a general membrane abnormality. The postulated membrane changes were subsequently substantiated by electron spin resonance studies. Fresh myotonic red blood cells demonstrated an increased fluidity and a decreased polarity compared to control red blood cells [5].

Since this sensitive biophysical technique revealed an abnormality in fresh erythrocytes, it appeared reasonable that protein phosphorylation might be impaired under similar conditions. Such a demonstration was not possible with our prior method of preparing erythrocytes, and an alternative technique of ghost preparation was sought. Our present methods eliminated magnesium from the lysis solution to obtain more permeable membrane preparations [6, 13, 14, 26]. With this technique, phosphorylation of one of the membrane glycoproteins was significantly decreased in fresh myotonic

erythrocyte membranes. The reduced rate of phosphorylation of this component of myotonic membranes and characterization of the phosphorylated substrate represent the subject of the present report.

Materials and Methods

Erythrocyte ghosts were prepared by a hypotonic lysis method which used 5 mM sodium phosphate buffer, pH 8.0 [6]. Ghosts were freshly prepared and all assays of endogenous protein kinase activity were carried out as previously described except that no freezing or storage were employed [22]. The incubation mix contained 10 μmoles of sodium acetate buffer, pH 6.5, 2.0 μmoles of magnesium acetate, 0.06 μmoles of ethylene glycol bis (aminoethyl)-N,N′-tetraacetic acid, 1 nmole of [γ-^{32}P] ATP (3 to 5×10^6 cpm), and 200 μg ghost protein in a total volume of 0.20 ml. Reactions were performed in duplicate at 25 °C for 60 sec. The reaction was stopped and the membranes completely solubilized in 1% SDS, 0.5 mM ethylene diaminetetraacetic acid, and 0.6 mM mercaptoethanol, electrophoresed on 6% SDS-polyacrylamide gels, stained with Coomassie blue, cut into bands, solubilized and radioactivity determined by previously described methods [21]. Reproducibility of the assay was ± 5% on all duplicates included in the study. Additional duplicate experiments on the same sample (freshly prepared) agreed within 5%.

Extraction with lithium 3,5-diiodosalicylate (LIS) was performed by a modification of the method of Steck and Yu [27]. At a concentration of 36 mM LIS, the glycoproteins remain in the pellet. Both supernatant and pellet fractions were solubilized and electrophoresed on 6% SDS-polyacrylamide gels. Lengthening the time of extraction to one hour and washing in 10% tricholoroacetic acid-fixed gels in distilled water for several hours to remove LIS was employed to enhance the staining.

The major glycoprotein was extracted by the procedure of Hamaguchi and Cleve [11]. Periodic acid-Schiff (PAS) staining was performed by the method of Zacharius *et al.* [29]. Phospholipids and phosphorylated amino acids were determined as previously described [22]. ATPase was assayed by both the methods of Avruch and Fairbanks [2] and Wins and Schoffeniels [28]. Adenyl cyclase assays were kindly performed by Dr. Robert Lefkowitz [16, 18]. Protein concentrations were measured by the method of Lowry *et al.* [19]. Sialic acid was assayed by the method of Aminoff [1]. [γ-^{32}P] ATP was prepared by a modification of the method of Glynn and Chappell [7].

Results

Electrophoresis of control and myotonic ghost preparations revealed no differences in their polypeptide composition [22], and enzymatic assay of the erythrocyte ghosts revealed no differences in (Ca^{++}) ATPase, (Mg^{++}) ATPase, and (Na^+, K^+, Mg^{++}) ATPase activities (Table 1). Phosphoprotein phosphatase activity was undetectable and adenyl cyclase activity was too low to demonstrate any differences in both preparations. By electrophoresis polypeptide band III was present in the highest concentration and represented approximately 30% of the membrane protein (Fig. 1). Band III is not a single species but is known to represent the area of migration of several polypeptides including Component a [15, 17]. Band III refers to this

Table 1. ATPase activities of erythrocyte ghosts

	Control (9)	Myotonic (9)	p
(Mg^{++}) ATPase	0.133 ± 0.017	0.129 ± 0.015	NS
(Na^{+}, K^{+}, Mg^{++}) ATPase	0.238 ± 0.025	0.186 ± 0.026	NS
(Ca^{++}) ATPase	0.059 ± 0.013	0.061 ± 0.015	NS

ATPase is measured as μM PO_4/mg ghost protein/hr. (Na^{+}, K^{+}, Mg^{++}) ATPase measured as the difference between total (Na^{+}, K^{+}, Mg^{++}) ATPase and oubain (0.3 mM)-inhibited (Na^{+}, K^{+}, Mg^{++}) ATPase. Values are listed as the mean ± SEM. NS = No significant difference as determined by the Student's t test.

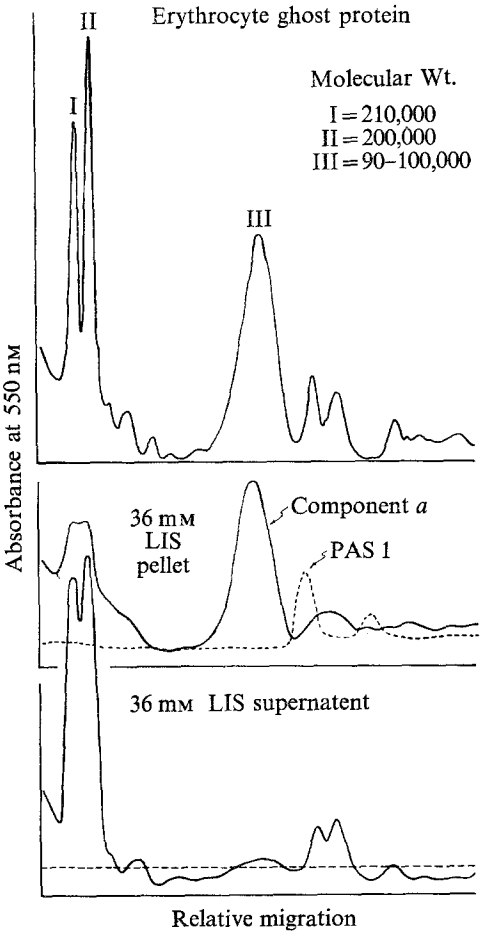

Fig. 1. Densitometry spectra of SDS-polyacrylamide gels stained with Coomassie blue (solid lines) and PAS (dotted lines). Top: Whole erythrocyte ghost; Middle: Pellet following extraction with 36 mM lithium diiodosalicylate; Bottom: Supernatant of 36 mM LIS extraction. There are no differences in the SDS-polyacrylamide gel pattern of myotonic and control ghosts. Band III region has the identical relative integrated stain intensity in myotonic and control ghosts [22]

Table 2. Phosphorylation of 90,000 to 100,000 molecular weight band III

Exp.	Control (pmoles/mg[a]/min)	Myotonic (pmoles/mg[a]/min)
1	2.22	1.28
2	2.68	0.794
3	3.62	3.52
4	3.77	3.16
5	3.08	1.89
6	2.92	2.31
7	1.87	1.99
8	2.82	2.17
9	3.45	2.61
10	2.24	1.81
11	2.87	1.61
12	2.35	1.70
Mean	2.82	2.07
SD	0.592	0.760
SEM	0.171	0.219
p	<0.02	

[a] mg refers to mg ghost protein.

stained band in nonextracted ghost preparations. Component a refers to the LIS-extracted residue bands that migrate with the same R_f as band III [27].

At 25 °C, phosphorylation of polypeptide III was linear for up to $3^1/_2$ min at ghost protein concentrations of up to 500 μg/assay in both myotonic and control preparations. Studies of the K_m for ATP yielded identical values of 8 μM for both myotonic and control preparations [10, 22, 24]. The assays described in this study were performed at 5 μM ATP to maximize incorporated radioactivity. Previous studies have demonstrated that this concentration of ATP was not limiting after a 5-min incubation period [10, 21] and all experiments herein reported were incubated for 1 min.

In myotonic erythrocytes, the phosphorylation of polypeptide III was decreased compared to control preparations ($p < 0.02$). Table 2 reports the data from 12 consecutive experiments in which nine different patients from five separate myotonic families participated. All but one patient were nonhospitalized. Controls consisted of ten age- and sex-matched, nonhospitalized volunteers. It should be noted that in experiment # 3 in Table 2 the blood sample from the myotonic patient was obtained one week after receiving six pints of whole blood for a pulmonary lobectomy. This value is the highest myotonic value noted. In experiment # 7, the control was low; and this same control had participated in experiment # 1 thereby giving rise to the two lowest control values. At 25 °C, phosphorylation of polypeptide II was suggestively, but not significantly, lower in the myotonic membranes.

Table 3. Phosphorylation of component a following LIS isolation procedure

Exp.	Myotonic (pmoles/mg [a]/min)	Control (pmoles/mg [a]/min)
I	0.620	1.40
II	0.479	1.64
III	0.341	1.64
IV	0.963	1.82
Va	1.36	1.97
b	1.03	
Mean	0.799	1.69
SD	0.384	0.215
SEM	0.156	0.096
p	<0.005	

[a] mg refers to mg LIS extracted residue protein.

To define the phosphorylated substrate, the glycoproteins were extracted from a phosphorylated ghost preparation using 36 mM lithium diiodosalicylate. According to Steck and Yu [27], the membrane glycoproteins including Component a are retained in the residue fraction by this procedure. In our own experiments, gel electrophoresis with staining for proteins and glycoproteins as well as direct assay of sialic acid and phospholipids confirmed this localization.

The extent of phosphorylation of various components of both supernatant and residue fractions was examined. Phosphorylation of Component a was found only in the residue fraction, and migrated at a position on the gels analogous to the migration of polypeptide III of whole ghosts prior to fractionation (Fig. 1). In the supernatant fraction no bands were phosphorylated which migrated in the same region as polypeptide III (namely, the 90 to 100,000 mol wt region). Phosphorylation of the 200,000 mol wt polypeptide was demonstrable principally in the supernatant fraction and was not significantly different. In residue fractions Component a was significantly less phosphorylated in myotonic patients than in control preparations (Table 3).

In order to assure that Component a and not the major sialoglycoprotein was the site of phosphorylation, the major sialoglycoprotein of the erythrocyte was extracted by the method of Hamaguchi and Cleve [11]. In this procedure Component a is not extracted; but the major glycoprotein is removed and can be identified by electrophoresis on acrylamide gels according to its anomalous migration as a polypeptide with an apparent molecular weight of about 75,000. No radioactivity could be demonstrated in the

extracted major glycoprotein, and all phosphorylation was retained in the precipitate. The major sialoglycoprotein comigrated with the major sialoglycoprotein obtained by LIS extraction (Fig. 1).

Discussion

Erythrocyte ghosts demonstrate considerable variation in enzymatic activity depending upon the technique employed to prepare the ghosts [12]. In our previous study using ghosts prepared by hypotonic lysis in the presence of 1 mM $MgCl_2$ at pH 7.4, we found that freezing at -20 °C for one week was necessary to demonstrate differences in endogenous protein kinase activity. Minimal differences were noted in fresh ghosts [22]. In the present study we used ghosts which were initially lysed and washed in the absence of Mg^{++}. The exact structural changes induced by the absence of Mg^{++} are unclear, but the ability to demonstrate differences between control and myotonic preparations may be related to the enhanced permeability of Mg^{++}-free ghosts [13, 14, 26]. Addition of Mg^{++} to the lysis solution has been demonstrated to affect physical as well as biochemical characteristics of ghosts. Using protein magnetic resonance spectroscopy Sheety and Chan demonstrated that 0.5 mM Mg^{++} will stabilize ghost proteins and eliminate the high resolution pmr signal [25].

Component a of the erythrocyte was the principle substrate of phosphorylation in the 90 to 100,000 mol wt region of the gel. This protein contains a small amount of carbohydrate (5 to 8% by weight) [8] and represents approximately 30% of total membrane protein [6, 9, 21]. The diminished phosphorylation of myotonic component III (which contains Component a) that was demonstrated in the whole ghost preparations has been reproduced in residue fractions following LIS extraction. With these techniques Component a from myotonic membranes is phosphorylated approximately 50% of control.

It is still unclear whether the difference in endogenous protein kinase activity demonstrated in myotonic ghosts is due to an intrinsic difference in the protein kinase enzyme or its substrate, or whether the result is due to a change in the state of the membrane. It is of interest that activity of two other endogenous enzymes, (Na^+, K^+, Mg^{++}) ATPase and (Ca^{++}) ATPase, were the same in normal and myotonic preparations. Electron spin resonance studies of red blood cells from myotonic patients have demonstrated an increased fluidity and a less polar spin label environment supporting differences in lipid-lipid or lipid-protein interactions within the membrane [5]. Reduced phosphorylation of Component a in the myotonic may have several explanations: (1) Component a may exist in a more

phosphorylated state and may not be additionally phosphorylated; (2) the primary structure of Component a may be different in myotonic membranes; (3) the protein substrate may be altered by its membrane environment so as to reduce the sites available for phosphorylation; or (4) the protein kinase enzyme activity may be directly or indirectly altered.

Direct or indirect alterations in Component a would be in accord with changes in membrane fluidity or polarity since this protein is one of two glycoproteins that spans the entire membrane. By labeling studies with ^{35}S-formylmethionyl sulfate methyl phosphate, Bretscher has demonstrated that Component a is exposed on both inner and outer surfaces [3]. Phillips and Morrison have confirmed this conclusion using ^{125}I and lacto-peroxidase labeling techniques [20]. Changes of intramembrane lipid-lipid or lipid-protein relationships could affect the availability of active sites for phosphorylation of this intrinsic glycoprotein that is strongly bound to the membrane and resists attempts to remove it from the lipid components [3, 6]. Bretscher has also postulated a specific catalytic role for Component a suggesting that it may be an anion channel [4]. Whether alterations in the phosphorylated polypeptides of membranes are responsible for the physiological changes of myotonic cells is not clear. However, it is clear from the present data that fresh red cell membranes exhibit a membrane abnormality and that such membrane alterations may be widespread in myotonic muscular dystrophy [23].

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