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Phosphoprotein Phosphatase Activity in Normal and Dystonic Human Fibroblast Plasma Membranes (41082)

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Abstract. There is increasing evidence that the level of membrane protein phosphorylation may be related to the regulation of membrane function. We have characterized the phosphoprotein phosphatase activity in membranes prepared from normal human skin fibroblast cultures and from patients with dystonia musculorum deformans (DMD). Protein phosphatase activity was linear with time and membrane protein with a broad pH optimum between 6.0 and 7.5. Fluoride, molybdate, and compounds containing a pyrophosphate moiety were inhibitory. There was no significant difference in the K_m or V_{max} when fibroblast membranes from patients with DMD were compared to those from normal controls.

Endogenous membrane protein phosphorylation is demonstrable in a wide variety of membranes from different organs and tissues (1) and is thought to play a major role in regulating membrane function (2). Several studies suggest that membrane protein phosphorylation is related to ion permeability (2, 3). In addition, more recent reports suggest that membrane phosphorylation may be a key biochemical mechanism for mediating or regulating the function of specialized receptor proteins in the cell membrane. Thus, insulin (4), epidermal growth factor (5), and fibroblast growth factor (6) stimulate phosphorylation of specific membrane proteins in their target cells. Serum-stimulated growth in normal 3T3 fibroblasts is also associated with an increase in membrane protein kinase activity (7). These data suggest that the activity of specialized membrane proteins may be regulated by reversible phosphorylation–dephosphorylation reactions. Indeed, we have shown that the membrane-bound nicotinic acetylcholine receptor is a specific substrate for a reversible phosphorylation–dephosphorylation reaction which is regulated by membrane kinase and phosphatase activity (8). These findings suggest that soon it may be possible to correlate the level of membrane phosphorylation with the function of specific membrane proteins. Therefore, it may be anticipated that abnormalities in membrane phosphorylation or dephosphorylation will be discovered in patients with inherited disorders of membrane function.

Membrane protein phosphatase activity is as important as protein kinase activity in determining the level of membrane protein phosphorylation. However, less is known about the regulation and significance of membrane protein phosphatase activity. In particular, there are no reports in the literature about membrane protein phosphatase activity in human fibroblast cell lines. Since these cultures are commonly used to investigate the metabolic basis of inherited disease, we present this report to characterize the properties of protein phosphatase activity in membranes prepared from normal human skin fibroblast cell cultures and from the fibroblasts of patients with dystonia musculorum deformans.

Materials and Methods. *Membrane preparation from human skin fibroblasts.* Kartner *et al.* (9) recently described a convenient and reproducible method to prepare isolated plasma membranes from human skin fibroblasts. The method in their paper was followed with little modification. Human skin fibroblast cultures, HS-27F, were grown to confluency in 500-cm^2 area roller bottles containing RMPI 1640 medium supplemented with 10% fetal calf serum. The cells were washed five or six times with 25 ml Dulbecco's phosphate-

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buffered saline (PBS) at room temperature and once with 25 ml ice-cold 1 m*M* $NaHCO_3$, pH 7.4, to remove excess PBS. The washed cells were swollen at room temperature by slowly rotating the bottles containing 25 ml of the hypotonic medium. The swollen cells could be completely removed from the roller bottles and disrupted by manual rapping and gentle swirling with two washes.

EDTA was then added to the disrupted cell suspension to a final concentration of 0.5 m*M* and the suspension was cooled in ice for 10 min. This procedure prevents aggregation and clumping of the membrane particles. The suspension was then centrifuged at 27,000*g* for 20 min and the pellet was resuspended through a 26-gauge needle in 10 ml of 10% (w/v) sucrose. The suspension was layered on a discontinuous density gradient composed of 15 ml each of 30, 48, and 60% (w/v) sucrose and centrifuged at 68,000*g* for 2.5 hr in an SW 25.2 rotor (Beckman). The gradient was harvested with a Buchler auto densiflow apparatus and the fraction at the 10–30% interface was used for subsequent study. We confirmed the results of Kartner *et al.* (8) that this fraction is markedly enriched in 5′-nucleotidase activity.

Skin fibroblasts from patients with the recessive and dominant forms of DMD and age- and sex-matched controls were obtained from the Human Genetic Mutant Cell Repository in Camden, New Jersey.

Preparation of $(^{32}P)PO_4$ *casein.* Commercially available casein (Miles Laboratories, purified grade) was pretreated by heating 25 mg/ml in 1 m*M* Hepes, 1 m*M* EDTA, pH 8.0, at 70° for 20 min. The solution was dialyzed against several changes of buffer at 4°. Protein concentration of the dialysate was approximately 12 mg/ml.

In order to prepare $[^{32}P]PO_4$ casein of high specific radioactivity the naturally occurring phosphate linkages were first cleaved by a phosphoprotein phosphatase activity associated with an insoluble crude membrane preparation. We have found that membranes from the electric organ of the ray *Torpedo californica* have significant phosphoprotein phosphatase activity (10). These membranes were prepared as described (11) and resuspended in 15 m*M* Tris–HCl, pH 6.8. Dephosphorylation of casein was carried out in a mixture containing 36 mg crude *Torpedo* membrane protein, 72 mg pretreated casein, 4 m*M* DTT, and 15 m*M* Tris–HCl, pH 6.8, in a total volume of 8.1 ml. The reaction was incubated for 90 min at 37° in a water bath shaker and terminated by centrifugation at 48,000*g* for 60 min. After exhaustive dialysis of the supernatant against 15 m*M* Tris–HCl, pH 6.8, the dephosphocasein was stored frozen at a concentration of 9–10 mg/ml.

The dephosphorylated casein could now be phosphorylated with a radioactive label by the membrane protein kinase present in the acetylcholine receptor-enriched membranes from *T. californica* which had been aged by storage at −20° for at least 3 months. Under these conditions the aged membranes lose phosphoprotein phosphatase activity (Gordon and Diamond, unpublished observations). Casein was phosphorylated in a mixture containing 9 mg/ml dephosphocasein, 0.5 μM [γ-^{32}P]ATP (18 μCi) (New England Nuclear), 9 mg/ml receptor-enriched membranes, 0.25 m*M* ouabain, 0.25 m*M* ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 0.005% Triton X-100, 5 m*M* $MnCl_2$, 100 m*M* KF, and 15 m*M* Tris–HCl, pH 6.8, in a total volume of 6.1 ml and incubated at 37° for 1 hr. Phosphorylation of casein was terminated by centrifugation at 48,000*g* for 1 hr. The supernatant was exhaustively dialyzed at 4° against several changes of 15 m*M* Tris–HCl, pH 6.8, to remove the unreacted [γ-^{32}P]ATP and any $[^{32}P]PO_4$ present. The retentate was applied to a 1.5 × 25-cm P2 (Bio-Rad) column previously equilibrated with 15 m*M* Tris–HCl, pH 6.8. Eluate fractions, 0.8 ml, were monitored for $(^{32}P)PO_4$ by Cerenkov counting of 10-μl aliquots in minivials with 3 ml H_2O in a Beckman LS 233 scintillation counter. Samples in the void volume peak containing $[^{32}P]PO_4$ casein were pooled and again exhaustively dialyzed at 4° against several changes of 15 m*M* Tris–HCl, pH 6.8, to lower background in the phosphatase assay (see below). The final specific radioactivity of $[^{32}P]PO_4$ casein was typically about 120,000 cpm/mg. Protein was measured as described by Lowry *et al.* (12).

Phosphoprotein phosphatase assay. Human skin fibroblast plasma membrane phosphoprotein phosphatase activity was determined by a modification of the method of Graham *et al.* (13). Membranes containing 5–10 μg of protein were incubated in duplicate with 100 μg [^{32}P]PO_4 casein at 37° for 20 or 30 min in 100 μl incubation mixture containing 4 m*M* DTT, 0.1% Triton X-100, and 15 m*M* Tris–HCl, pH 6.8. The reaction was terminated by addition of 3 ml of an ice-cold suspension of 40 mg/ml activated charcoal (Norit A), 0.1 *M* HCl, 0.2 mg/ml bovine serum albumin, and 1 m*M* each of sodium orthophosphate and pyrophosphate. After holding on ice for 10 min the samples were either frozen for later analysis or assayed immediately as previously described (13). All data were corrected by subtraction of blank values obtained by simultaneous incubation of [^{32}P]PO_4 casein in the absence of membranes or in the presence of boiled membrane controls.

Radioactive [^{32}P]PO_4 released from casein after incubation with membranes could be due to proteolytic breakdown of casein into labeled amino acids or pepetides. In order to confirm that the measured radioactivity was due only to [^{32}P]PO_4, the reaction products were also complexed with acidified molybdate and extracted into isobutanol (13). We found that radioactivity released from casein was extractable confirming that the label was inorganic phosphate.

Results. Our results indicate that phosphoprotein phosphatase activity is demonstrable in human skin fibroblast plasma membranes when measured with phosphorylated casein as a substrate. Figure 1 shows the kinetics of fibroblast plasma

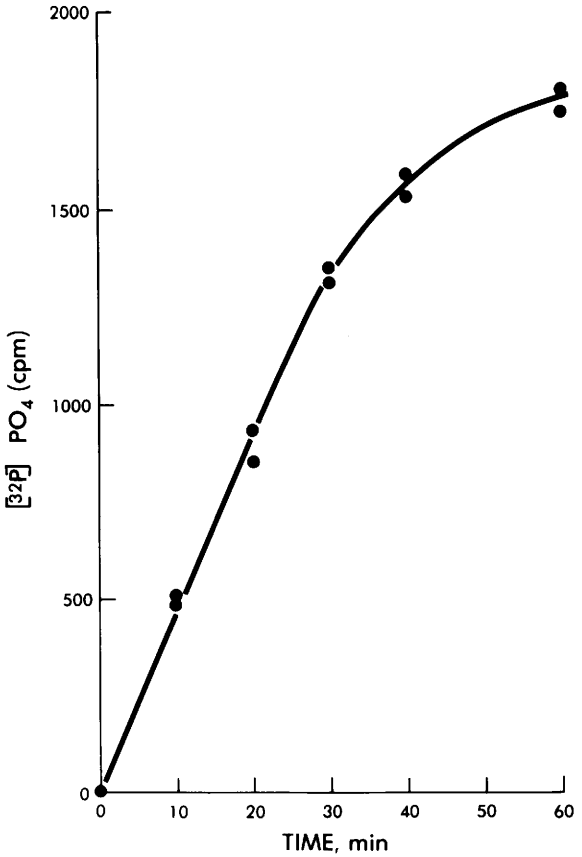

FIG. 1. Time course of dephosphorylation of [^{32}P]PO_4 casein by human skin fibroblasts plasma membrane. Plasma membranes (10 μg) were incubated with [^{32}P]PO_4 casein (1.06 mg/ml) as described under Materials and Methods. Activity is expressed as cpm of [^{32}P]PO_4 released minus background release in the absence of membranes.

membrane-catalyzed release of $[^{32}P]PO_4$ from $[^{32}P]PO_4$ casein obtained under standard conditions of assay. The rate of $[^{32}P]PO_4$ release was linear up to 30 min when approximately 75% of the releasable phosphate had been released. Maximal release of $[^{32}P]PO_4$ typically ranged from 7 to 10% of the total radioactivity bound to $[^{32}P]PO_4$–casein with all preparations employed. Figure 2 demonstrates that release of $[^{32}P]PO_4$ from the substrate was proportional to the concentration of membrane protein up to 20 μg of fibroblast membranes. The pH activity profile (Fig. 3) shows a broad pH optimum between pH 6.0 and 7.5. Below pH 5 casein precipitates from solution and becomes unavailable to the membrane phosphatase. Activity declines sharply above pH 8 indicating that the phosphoprotein phosphatase in human skin fibroblast membranes is not an alkaline phosphatase.

The dependence of membrane phosphatase activity on substrate concentration is depicted in Fig. 4. The inset shows a double-reciprocal plot used to determine the kinetic parameters. The K_m and V_{max} were 0.71 mg/ml $[^{32}P]PO_4$ casein and 1625 cpm $[^{32}P]PO_4$, respectively.

The activity of the fibroblast plasma membrane phosphoprotein phosphatase was affected by several substances (Table I). Both sodium and potassium ions were mildly stimulatory at 10 m*M*; enzyme activity was inhibited at higher salt concentrations. The divalent cations Mg^{2+} and Ca^{2+} stimulated activity about 30% at a 1 m*M* concentration of the chloride salt, but

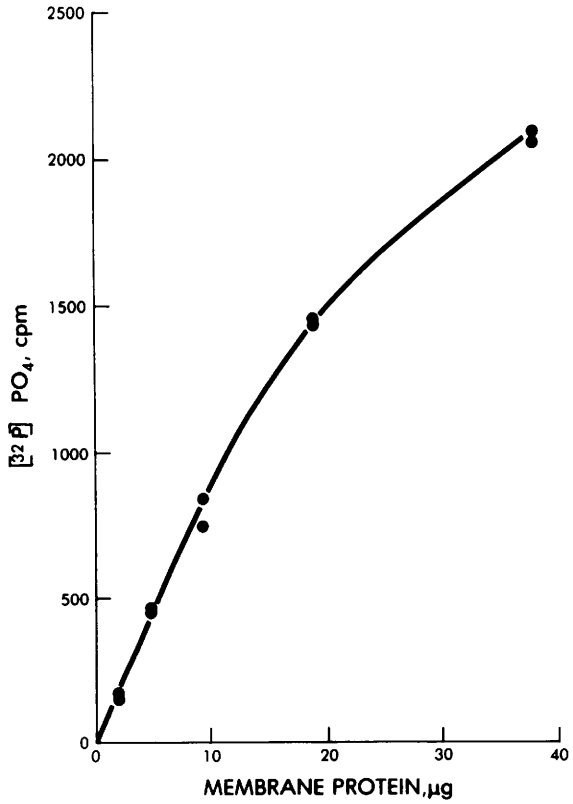

FIG. 2. Dephosphorylation of $[^{32}P]PO_4$ casein by human skin fibroblast plasma membranes as a function of membrane protein concentration. Plasma membranes were incubated with $[^{32}P]PO_4$ casein (1.06 mg/ml) for 20 min as described under Materials and Methods. Background activity in the absence of membranes has been subtracted.

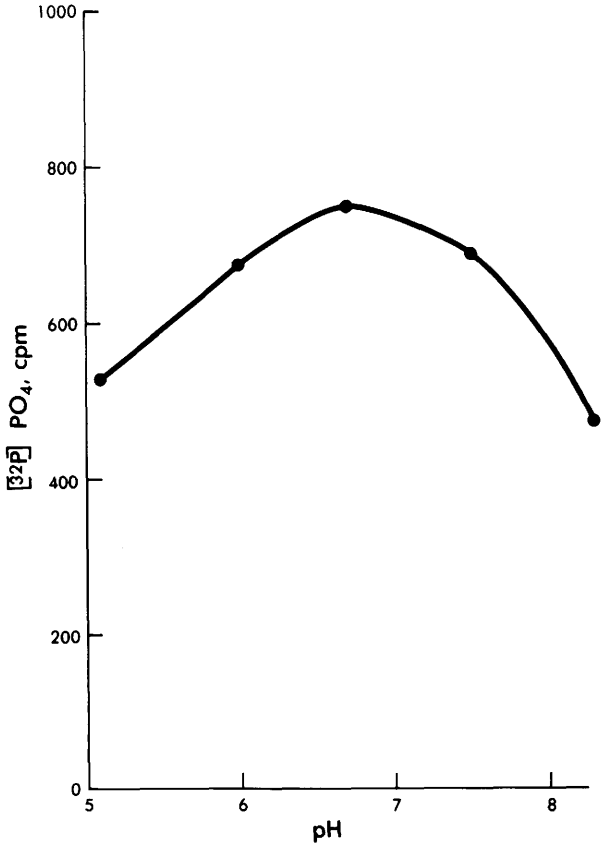

FIG. 3. Phosphoprotein phosphatase activity of human skin fibroblast plasma membranes as a function of pH. Buffer systems were: pH 5.1–15 m*M* 2-[*N*-morpholino]ethanesulfonic acid–Tris–HCl; pH 6.0–15 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid–Tris–HCl; pH 6.7, 7.5, 8.3–15 m*M* Tris–HCl. Counts per minute corrected for background activity in the absence of membranes.

Zn^{2+} consistently inhibited enzymatic activity. Fluoride inhibited the phosphatase equally at 10 and 100 m*M*. Compounds containing phosphate were, in general, inhibitory. Especially striking was the potent inhibition exerted by substances containing the pyrophosphoryl moiety such as ATP, GTP, and sodium pyrophosphate. Physiologic concentrations of cAMP and cGMP did not affect membrane phosphatase activity. Molybdate ion, a structural analog of the phosphate ion, completely abolished phosphatase activity at 100 m*M* concentration.

The biochemical defect in DMD is unknown. Preliminary evidence has been presented to suggest that fibroblasts from patients with DMD might have a disorder of amino acid transport (14). Since a defect in the regulation of membrane function might explain some of the reversible symptoms in this disorder, we investigated membrane phosphoprotein phosphatase activity in fibroblasts from patients with the dominant or recessive form of this disease. Figure 5 shows that there was no significant difference in the kinetics of membrane phosphatase activity when DMD fibroblast membranes were compared with those from age- and sex-matched controls. This was confirmed in a total of six paired experiments. Moreover, there was no apparent difference in DMD membrane phosphoprotein phosphatase sensitivity when a vari-

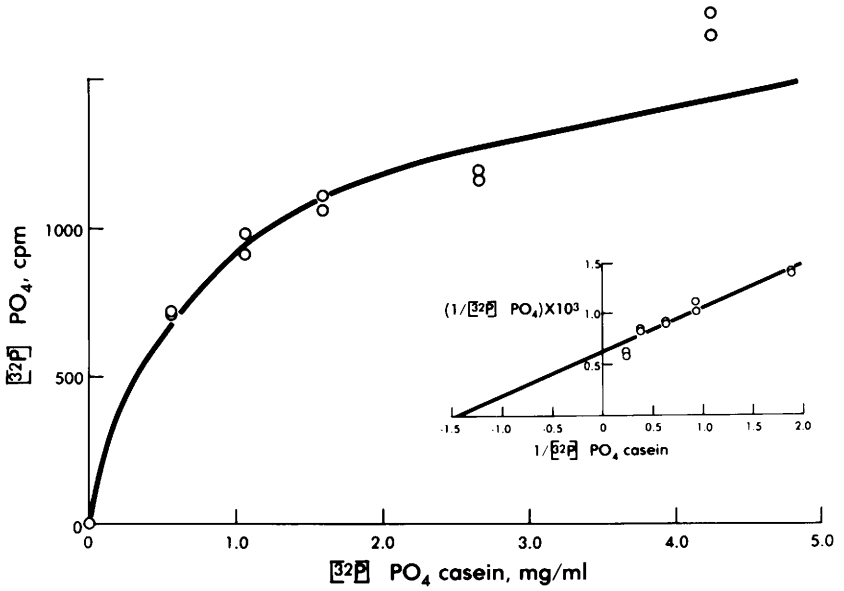

FIG. 4. Phosphoprotein phosphatase activity as a function of [^{32}P]PO_4 casein concentration. Plasma membranes (10 μg) were incubated with the indicated amount of [^{32}P]PO_4 casein for 20 min as described under Materials and Methods. The inset is a double-reciprocal plot of the data.

ety of metabolic agents were used to inhibit phosphatase activity.

Discussion. Addition of serum to normal or transformed mouse fibroblasts causes a rapid and striking decrease in cell surface phosphoprotein phosphatase activity (15). This finding suggests that plasma membrane phosphatase activity is under metabolic regulation and should be considered as a potential site for abnormality in suspected disorders of membrane function in man. The data reported in this study provide the first description of membrane phosphoprotein phosphatase activity in human skin fibroblast cultures. Membrane phosphatase activity was linear with time and membrane protein and not due to alkaline phosphatase activity. Rabbit liver membranes (16) and mouse fibroblasts (17) have also been used to study membrane-bound phosphoprotein phosphatase activity with soluble substrates. Our results are very similar to results found with these latter preparations. Human fibroblast membrane phosphatase activity was unaffected by cyclic nucleotides but inhibited substantially by reagents containing a pyrophosphoryl moiety. ATP, GTP, and sodium pyrophosphate were potent inhibitors of the membrane phosphoprotein phosphatase and molybdate ion, which is a structural analog of phosphate ion, completely inhibited enzyme activity at 100 m*M*. This suggests that molybdate might be useful to block phosphatase activity in studies of the function of phosphorylated proteins *in vitro*.

Since it is exceedingly difficult to study the regulation of membrane phosphatase activity with endogenous membrane proteins as substrates, we have used an assay that depends on the dephosphorylation of exogenously added phosphorylated casein. This approach has been useful in recent reports of membrane protein kinase and phosphatase activities. In such well-defined systems the phosphatase is examined independently from the kinase and the reaction product is easily separated from the membrane-bound enzyme. This makes it possible to generate reliable data for the membrane protein phosphatase kinetic parameters, K_m and V_{max}. In addition, the assay is suitable for screening human fibroblast membranes taken from patients with suspected membrane disorders. In prelimi-

TABLE I. EFFECT OF IONS AND METABOLITES ON PLASMA MEMBRANE PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

Addition	Concentration (m*M*)	Relative activity (%)	Addition	Concentration (m*M*)	Relative activity (%)
None	—	100			
NaCl	1	98	ATP	0.01	77
	10	113		0.1	67
	100	65		1	25
KCl	1	96	GTP	0.01	76
	10	105		0.1	77
	100	57		1	19
NaF	1	96	NaP_i	0.1	98
	10	37		1	68
	100	37		10	44
$CaCl_2$	0.1				
	1	101	$NaPP_i$	0.1	56
		131		1	20
				10	5
$MgCl_2$	0.1	93			
	1	127	cAMP, cGMP	0.001	100
$ZnCl_2$	0.1	76	$NaMoO_4$	1	77
	1	48		10	44
				100	0

Note. Human skin fibroblast phosphoprotein activity was assayed by incubating plasma membranes (10 μg) with [^{32}P]PO_4 casein (1.06 mg/ml) for 20 min as described under Materials and Methods. Activity for each effector is calculated relative to no additions (100%).

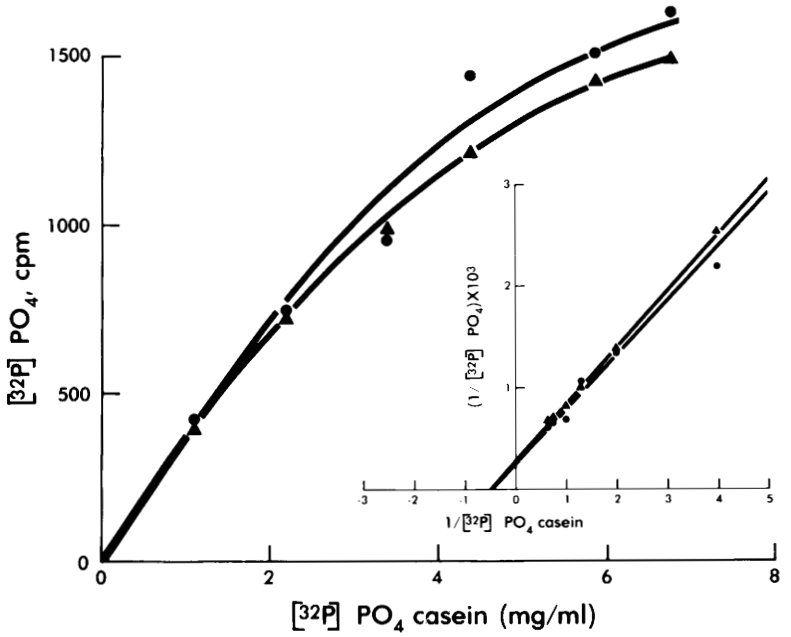

FIG. 5. Comparison of phosphoprotein activity as a function of [^{32}P]PO_4 casein concentration for normal human skin fibroblasts and fibroblasts grown from a patient with the recessive form of DMD. ○, normal human skin fibroblast (GM 2912) plasma membranes; human skin fibroblast plasma membranes from a patient with a recessive form of DMD (GM 3218).

nary studies, fibroblasts from patients with DMD were thought to show a possible disorder of membrane transport (14). Therefore we screened fibroblast membrane preparations from patients with DMD to search for significant differences in their membrane phosphoprotein phosphatase activities. However, we were unable to find an abnormality of this membrane-dependent enzyme system using an exogenous substrate. We are now developing methods to study phosphoprotein phosphatase activity using endogenous substrates which may be more sensitive to detecting defects in membrane structure and function.

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