DEFECTIVE PROTEIN PHOSPHORYLATION IN MEMBRANES OF HEREDITARY SPHEROCYTOSIS ERYTHROCYTES

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1. Introduction

The presence of cyclic AMP dependent protein kinases and polypeptide substrates has been demonstrated in a number of membranes [1]. The operation of these kinases and substrates may have important membrane regulatory functions although specific demonstration of altered membrane properties dependent on phosphorylation of a membrane polypeptide has not been established. It has been suggested that this phosphorylation reaction may regulate ion transport in muscle and synaptic membrane [2,3].

The erythrocyte membrane also contains a protein kinase and polypeptide substrates [4] including polypeptides which may have a structural role in the membrane [5]. We have examined this reaction in erythrocytes with a hereditary defect in cell shape as an initial study in defining the effect of membrane protein phosphorylation on membrane shape and permeability regulation. The current study demonstrates a phosphorylation defect in hereditary spherocytosis (HS) membranes which is observed in fresh membrane preparations. This decreased phosphorylation is due to defect in protein kinase activity and not due to reduced or defective substrate.

2. Experimental

Erythrocyte membrane was isolated by the procedure of Dodge et al. [6] except that cells were hemolyzed and washed in 0.01 M Tris–HCl, pH 7.4. The 0.5 ml reaction mixture contained 0.3 mg of membrane protein which was frozen and thawed prior to use, 0.15 mM of γ-^{32}P-labeled ATP (~ 20 cpm/pmole), 2.5 mM Mg^{2+} and 0.01 M Tris–HCl at pH 7.4. After incubation for 1 hr at 37°C, the reaction was stopped with the addition of 2 ml of ice cold 0.01 M Tris–HCl, 0.05 M NaCl, 1 mM EDTA at pH 7.4 and the suspension was centrifuged at 20 000 *g* for ten min. The membranes were then washed three times with this buffer.

Separation of the membrane polypeptides was accomplished after dissolving the sample in 1% SDS, 0.01 M dithiothreitol and electrophoresis in 5% polyacrylamide gels (SDS PAGE) as described by Weber and Osborn [7]. The band designations were assigned according to Fairbanks et al. [8]. Frozen gels were sliced in 1 or 2 mm segments, transferred to scintillation vials, dissolved in 30% H_2O_2, and counted by Cerenkov radiation in a Packard Model 3341 scintillation counter.

3. Results and discussion

Incorporation of label into erythrocyte membrane in the absence of adenosine 3′,5′-monophosphate (cAMP) is shown in fig. 1. The results are similar to previously described studies [4] except for the observation of nearly equivalent incorporation into both components of the high mol. wt. doublet (bands I and II which are collectively termed spectrin [5]). Equivalent uptake into each of these components was confirmed by direct slicing of each band from a 3% polyacrylamide gel after staining with Coomassie Blue. The favored incorporation into band II of spectrin reported previously [4] may be due to the different reaction conditions utilized by these groups. Additionally,

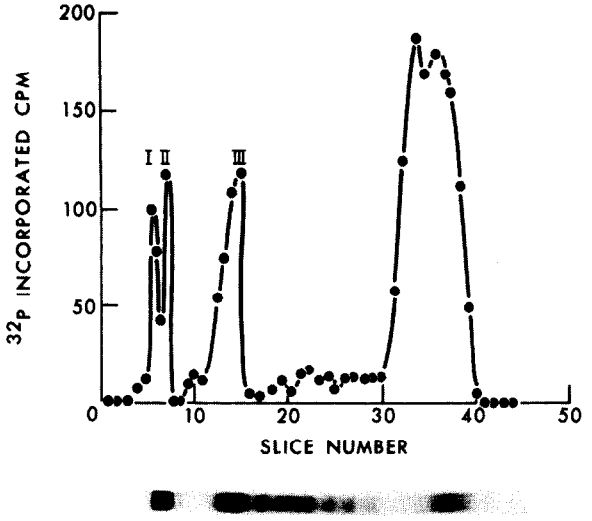

Fig. 1. ^{32}P-labelling profile of normal erythrocyte membrane phosphorylated as described in table 1 without cyclic AMP and resolved by electrophoresis on SDS polyacrylamide gels (5%). A 50 μg sample was applied and gel segments of 1 mm were obtained through bands I and II and 2 mm segments thereafter. A corresponding gel, containing 75 μg of membrane protein was stained with Coomassie Blue and is shown below the profile.

resolution of the two components is considerably improved by electrophoresis in 3% polyacrylamide gels. Under these conditions, each component appears to be equally accessible to the kinase and no functional difference is suggested by their behavior in the phosphorylation reaction.

The third peak for phosphorylation occurs in the 90 000 mol. wt. area of the gel (band III) which contains the principal glycoprotein and a second major intrinsic membrane protein [9]. Whether one or both are substrates has not been determined. The fourth peak contains residual ATP and labeled polyphosphoinositides [10].

In contrast to normal biconcave erythrocytes, erythrocytes from HS patients approach, to various extents, a spherical shape. They also have a decreased membrane deformability [11], enhanced passive permeability to sodium ion and a marked increase in osmotic fragility particularly after in vitro incubation for 24 hr. Clinically, the defective erythrocyte is susceptible to premature removal in the spleen which reduces cell survival and can result in anemia [12].

Except for variable elevations of residual hemoglobin, analyses of HS membrane polypeptides by SDS PAGE give staining patterns qualitatively and quantitatively indistinguishable from normal. However, the extent of phosphorylation of the membrane protein substrates of the kinase are markedly decreased as shown in table 1 ranging from 26 to 46% of controls. Although cyclic AMP can stimulate incorporation into HS membranes, the level is still depressed and lower than incorporation into normal preparations in the absence of cyclic AMP. The defect is present in both splenectomized and non-splenectomized patients and is not correlated with reticulocyte count. Since several different polypeptides are affected, this suggests that the primary defect is involved with the kinase enzyme rather than an unlikely simultaneous defect in the different polypeptide substrates.

Since the restoration of a permeability barrier by

Table 1
Phosphorylation of hereditary spherocytosis membrane proteins in the presence and absence of cyclic AMP

Sample	Bands I and II		Band III	
	No cAMP	cAMP	No cAMP	cAMP
Normals*	100 (± 5)	100	100 (± 4)	100
HS Membrane				
FT	26	45	31	26
AT	34	45	68	63
JE	40	54	45	42
NB	46	–	–	–

Numbers represent the percentage of normal membrane protein labelled and are the mean of three assays (standard errors of the mean are less than 12%). The incubation was performed at 37°C for one hr with 0.3 mg of membrane protein in the presence of 2.5 mM $MgCl_2$, 0.15 mM [^{32}P]-ATP (20 cpm per pmole), 0.01 M Tris (hydroxymethyl)–aminomethane–HCl, pH 7.4 with 1.0 μM cyclic AMP when required. Polypeptide bands were separated by SDS polyacrylamide gel electrophoresis.

* Incorporation into bands I and II was 154 pmoles/mg without cAMP and 202 pmoles/mg with cAMP; incorporation into band III was 140 pmoles/mg without cAMP and 202 pmoles/mg with cAMP. Patient FT, splenectomized with a reticulocyte count of 4%; AT, nonsplenectomized with a reticulocyte count of 13%; JE, nonsplenectomized with a reticulocyte count of 8%; NB, nonsplenectomized. The standard deviation for nine normal control is indicated in parenthesis.

membrane resealing can occur, the possibility of an enhanced resealing of the HS membrane effecting the incorporation reaction by a partial exclusion of reactants required consideration. The spectrin bands (I and II) are localized on the inner aspect of the membrane [9] and hence are inaccessible to the action of trypsin in intact and resealed cells [13]. Accordingly, both normal and HS membrane were exposed to trypsin (50: 1 w/w) for 1 hr at 37°C. The reaction was stopped with the addition of soy bean trypsin inhibitor (SBTI) and the products were examined by SDS PAGE. An unincubated normal containing both trypsin and SBTI was run as a control and showed no degradation. These conditions virtually eliminated bands I and II in both normal and HS membrane demonstrating these ghosts were freely permeable.

The present studies suggest that in the erythrocyte, phosphorylation may be important in the control of cell shape and deformability. The cell abnormalities in HS could be mediated through a conformational change in membrane proteins which results from failure of the kinase to maintain them in the phosphorylated state. Although, as suggested by Jacob [12], a defect in microfilamentous (or structural) protein may be crucial in generating the pathophysiological differences exhibited by HS cells, this defect may be secondary to the reduced protein kinase activity.

The decreased phosphorylation of HS membrane protein which we have demonstrated may result from altered or reduced levels of enzyme activity or from an altered interaction of the enzyme with its substrates. These distinctions are presently under investigation. The current results are consistent with a working hypothesis that phosphorylation of membrane protein is the energy dependent process responsible for the control of cell shape and deformability.

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