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CALCIUM-DEPENDENT ASSOCIATION OF GLUTATHIONE S-TRANSFERASE WITH THE HUMAN ERYTHROCYTE MEMBRANE

Patsy H. White and Gordon A. Plishker

Department of Neurology and the Program in Neuroscience,
Baylor College of Medicine, Houston TX 77030

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Elevations in intracellular calcium increase the adsorption of a cytoplasmic protein to human red blood cell membrane. This protein migrates on SDS polyacrylamide gels at 23,000 daltons and has been called band 8. The association of this protein with the membrane is increased in sickle cell anemia. This protein is extracted from the membrane with EGTA, a calcium chelator. Enzymatic and immunological studies identify band 8 as a glutathione S-transferase.

Calcium increases the adsorption of some cytoplasmic proteins with the human erythrocyte membrane. Hemoglobin, catalase (1) and carbonic anhydrase have all been shown previously to associate with the red cell membrane when the intracellular calcium concentration is raised.

In addition to these proteins, a 23,000 dalton protein, referred to as band 8 (2), has been observed, by us and others, to increase its association with the human red cell membrane in response to increased intracellular calcium (2,3). The membrane association of this protein is elevated in hereditary spherocytosis (2) and sickle cell anemia (3). Association of band 8 with the red cell membrane can be increased by incubation of energy depleted cells in calcium buffers, by use of A23187, a calcium ionophore, or by indirect inhibition of the Ca ATPase with trifluroperizine, which inhibits calmodulin (4).

We have determined that this protein is a glutathione S-transferase, one of a group of multifunctional, cytoplasmic proteins which catalyse the conjugation of glutathione with a wide variety of hydrophobic substrates having an electrophilic center (5). These enzymes are found predominately in the soluble fraction of the cell, although transferase activity has also been

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found in liver microsomes (6). The calcium-dependent association of these proteins with the plasma membrane has not been reported previously.

MATERIALS AND METHODS:

Blood from healthy volunteers and patients with sickle cell anemia was collected in heparinized syringes. The blood was centrifuged at 10,000 x g and the plasma and buffy coat were removed by aspiration. The cells were washed three times in normal saline and the membranes were prepared by the method of Fairbanks et al. (7). The membranes were solubilized and applied to a 10% acrylamide gel, which is 0.1% in SDS and subjected to electrophoresis as described by Laemelli (8). Protein was determined by the method of Lowry (9). Glutathione S-transferase activity was determined by the method of Habig et al. (5), except that the buffer was 100 mM imidazole, pH 6.5.

RESULTS:

The association of the 23,000 dalton band with the membrane is calcium dependent and is independent of the means taken to increase the intracellular calcium. Three examples of this are shown in Figure 1. Figure 1A shows red cell membranes prepared in a Tris-HCl buffer, pH 7.5, with or without magnesium or calcium. The membranes washed with buffer alone or with buffer containing 100 μM magnesium chloride do not show band 8, while those washed with buffer containing 10 or 100 μM calcium chloride show an increasing association of the band with the membrane. The effect of incubating cells preincubated with 4,4'-diisothiocyano-2,2'-stilbene sulfonate (DIDS) with or without calcium is shown in Figure 1B. Incubation of red cells with DIDS and calcium increased the intracellular calcium concentration from 16 ± 5 to 283 ± 25 μmoles / Lrbc. The amount of band 8 associated with the membrane was increased. DIDS alone did not increase the association of band 8 with the membrane. Figure 1C shows that, as compared to control, membranes prepared from red cells of a patient with sickle cell anemia had an increased amount of band 8. The sickle cells contained 103 ± 3 μmoles Ca / Lrbc; normal cells had 8 ± 1 μmoles Ca / Lrbc. This increased association of band 8 and elevated intracellular calcium was observed in 5/5 patients with sickle cell anemia.

Glutathione S-transferase could not be directly assayed in the membrane fraction because of light scattering at the wave-length of the assay. However, the proteins associated with the membranes in a calcium-dependent fashion could be eluted from the membrane with EGTA. Figure 2 shows a gel of

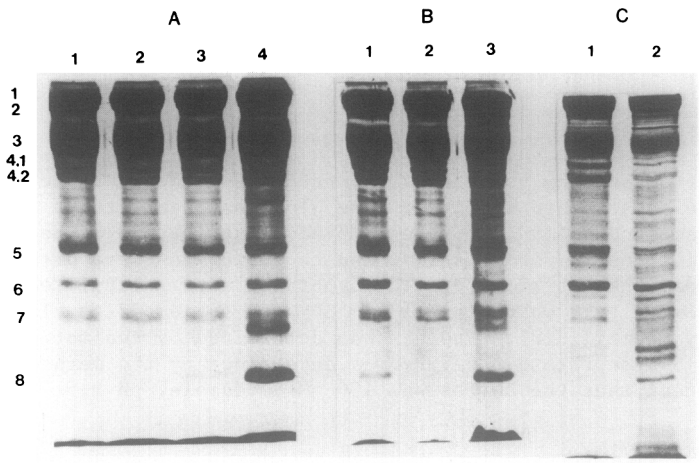

Figure 1. A picture of 0.1% SDS 10% polyacrylamide gels of red cell membranes showing the increase in association of band 8 with the membrane. Panel A shows a gel of membranes lysed and washed with or without $MgCl_2$ or $CaCl_2$. Cells washed as described in Materials and Methods were lysed and washed four times in 5 mM Tris-HCl, pH 7.5, with the following additions; Lane 1, nothing; Lane 2, 0.1 mM $MgCl_2$; Lane 3, 0.01 mM $CaCl_2$; Lane 4, 0.1 mM $CaCl_2$. Panel B shows a gel of DIDS treated cells. Red cells were preincubated with 0.3 mM DIDS, then washed 3 times in 0.9% saline. The cells were incubated for two hours in a buffer with or without 40 mM calcium. Lane 1, membranes from untreated cells; Lane 2, membranes from cells treated with DIDS and incubated without calcium; Lane 3, membranes from cells treated with DIDS and incubated with calcium. The cells incubated without calcium contained 16 ± 5 μmoles calcium / Lrbc. The cells incubated with calcium contained 283 ± 25 μmoles calcium / Lrbc. Panel C shows a gel comparing normal and sickle cell membranes. The membranes were prepared in 5 mM Tris-HCl, pH 7.5. Lane 1, membrane from normal cells; Lane 2, membranes from sickle cells. Calcium content of the normal cells was 8 ± 1 μmoles calcium / Lrbc. Calcium content of the sickle cells was 103 ± 3 μmoles calcium / Lrbc.

an EGTA extract of membranes which had been washed with calcium containing buffers as in Figure 1A. Band 8 is increased in both the membrane fraction and in the extract of the calcium washed ghosts. The specific and total activities of the glutathione S-transferase of these extracts are shown in Table 1. The total activity of glutathione S-transferase in the extract from the membranes washed with 100 M calcium chloride was 4.5 fold greater than control.

Immunological studies confirmed that the extracted band 8 was glutathione S-transferase. Antibodies were raised in white New Zealand rabbits against glutathione S-transferase purified from the hemolysate of human red blood cells. A positive antibody reaction was observed only with the 23,000 dalton band when proteins extracted by EGTA were subjected to electrophoresis and

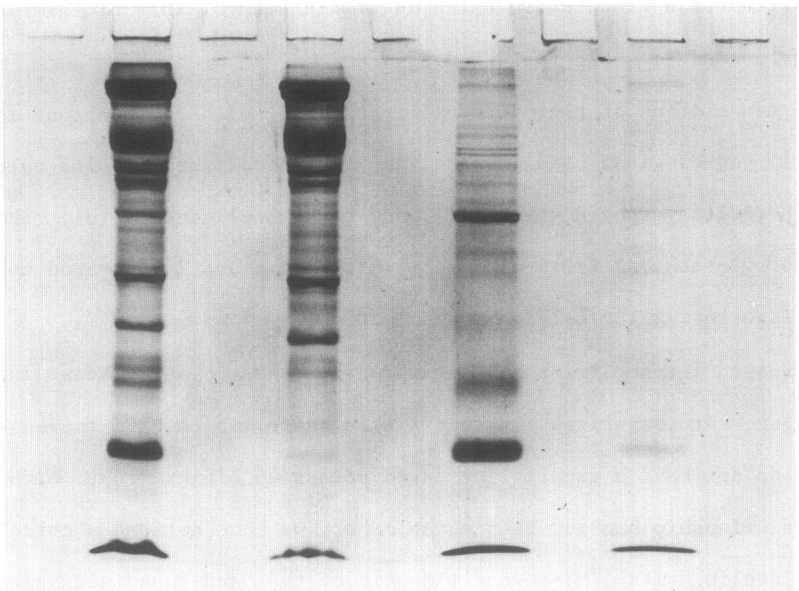

Figure 2. A picture of 0.1% SDS 10% polyacrylamide gel of red cell membranes, that were washed with and without calcium, and the EGTA extracts of these membranes. Normal red cells were lysed and washed with 5 mM Tris-HCl, pH 7.5 with or without 100 μM $CaCl_2$. Forty mls of packed ghosts were extracted with 40 ml of 5 mM EGTA at room temperature for 30 minutes. The ghosts were centrifuged at 30,000 x g for 10 minutes and the supernatants removed. Lane 1, membranes washed with 100 μM $CaCl_2$; Lane 2, membranes washed without calcium; Lane 3, EGTA extract of membranes shown in Lane 1; Lane 4, EGTA extract of membranes shown in Lane 2.

electroblotted onto nitrocellulose paper. The antibody also reacted with the 23,000 dalton band in the membrane.

DISCUSSION:

The cytoplasmic protein, which in the presence of calcium associates with the membranes and has a molecular weight of 23,000 on SDS polyacrylamide gels,

TABLE 1

GLUTATHIONE S-TRANSFERASE ACTIVITY IN EGTA EXTRACTS OF CALCIUM TREATED GHOSTS

	CONTROL	CALCIUM TREATED
Specific Activity	0.025 U/mg protein	0.043 U/mg protein
Total Activity	0.16 U	0.72 U

Glutathione S-transferase activity in the EGTA extract of membranes washed with or without calcium. Membranes were washed with or without 100 μM $CaCl_2$ as in Figure 2. EGTA extracts were made also as in Figure 2. The glutathione S-transferase activity was assayed by the method of Habig et al. One unit is one mole of 1-chloro 2,4-dinitrobenzene conjugated/minute.

is a glutathione S-transferase. The identification of this protein is based on enzymatic and immunological findings.

The association of glutathione S-transferase with the membrane is increased in pathological conditions having increased intracellular calcium, such as hereditary spherocytosis (2), and sickle cell anemia (3). The association of glutathione S-transferase with the membrane is elevated in normal cells by increasing the calcium concentration.

Increased intracellular calcium normally leads to an increase in potassium efflux. It is interesting that sickle cells, which have increased calcium, do not have a greatly increased potassium efflux (10). Although a causal relationship has not been established, we have noticed a correlation between labeling with [^{3}H]-iodoacetic acid of the band containing glutathione S-transferase and the inhibition of calcium-dependent potassium efflux (11).

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