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Ca^{2+}-mediated catabolism of human erythrocyte band 3 protein

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Catabolism of human erythrocyte membrane band 3 protein in the presence of Ca^{2+} was studied. An increase in the amount of a 30 kDa amino terminal fragment of band 3 was observed when erythrocyte membranes were incubated for 30 min with 1 mM Ca^{2+} in the presence of whole erythrosol. Incubation of the membranes with Ca^{2+} alone did not result in band 3 breakdown. Generation of the 30 kDa fragment from band 3 was related to the action of a leupeptin-sensitive Ca^{2+}-dependent proteinase in the cytosol. This proteinase was also responsible for the increased production of a 52 kDa and a 70 kDa transmembrane carboxyl terminal fragment of band 3. From the size of the generated fragments, it is deduced that in the presence of Ca^{2+} and Ca^{2+}-dependent proteinase, band 3 protein is cleaved at the cytoplasm/membrane interface and along its cytoplasmic domain.

Introduction

Band 3 is the anion transport protein of the human erythrocyte. The protein contains antigenic determinants important for blood group specificity [1,2]. Parts of the carboxyl terminal and transmembrane portions of band 3 are thought to constitute the senescent cell antigen, which is recognized by autologous antibodies directed against aged or abnormal red cells [3]. The amino terminal cytoplasmic domain of band 3, on the other hand, serves the important function of linking cytoplasmic proteins like hemoglobin and glycolytic enzymes as well as cytoskeletal elements to the erythrocyte membrane [4]. This 43 kDa cytoplasmic domain of band 3 can be released from erythrocyte membrane by treatment with proteinase like trypsin [4]. It is not known, however, whether catabolism of band 3 by intracellular proteinase occurs under certain cellular conditions with degradation of the cytoplasmic domain. If degradation does occur, it is expected that the interactions of band 3 with ankyrin, band 4.1 protein, hemoglobin and glycolytic enzymes would be seriously affected. In the present study, we examine the catabolism of band 3 protein mediated by Ca^{2+} to see whether the cytoplasmic domain of band 3 is degraded and to see whether any fragment related to the portion of band 3 containing the senescent cell antigen is generated. Catabolism of band 3 mediated by Ca^{2+} was studied because Ca^{2+} is implicated in a variety of pathophysiological red cell situations [5]. Besides, Ca^{2+} is required for the functioning of Ca^{2+}-dependent proteinase [6,7] which may be of importance in catabolism of band 3. Lorand et al. [5] and Lorand and Michalska [8] did show earlier that band 3 undergoes degradation in Ca^{2+}-loaded erythrocytes to give a protein with a molecular

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weight of approximately 55000, which they called 3′. Which portion of band 3 is represented by 3′, however, is not known.

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Materials and Methods

All chemicals used were of reagent grade. Ionophore A23187 was purchased from Sigma Chemical Co. (St. Louis, MO) and stock solution was made up in ethanol.

Preparation of erythrocyte membranes. Blood samples were collected in acid-citrate-dextrose from healthy donors and a band 3 variant reported by Morrison and coworkers [9,10] in the St. Jude Blood Bank Facility. Erythrocytes from freshly collected samples were passed through cellulose columns to ensure removal of white cells [11]. The red cells were washed three times with isotonic 0.155 M NaCl/3 mM histidine (pH 7.4) to remove plasma. The washed cells were then suspended at 10% hematocrit in the isotonic buffer and lysed by the addition of saponin (0.1 mg/ml) to the cell suspension. Membranes were sedimented by centrifugation at 40000 × *g* for 35 min and the supernatant was saved as the erythrosol fraction. The membranes were washed twice with the isotonic buffer containing 2 mM EDTA (pH 7.4). This was followed by two washes with the isotonic buffer to remove EDTA. The membranes were then further washed with 5 mM sodium phosphate buffer (pH 7.4), adjusted to about 1 mg protein/ml, and were kept at −70°C till use. Membranes thus prepared were open membranes as assessed by using the marker enzymes, Ca^{2+}-ATPase and acetylcholinesterase. They were resuspended in the incubation medium for digestion experiments.

Treatment of membranes. Membranes (350–400 μg protein) were incubated at 37°C in the presence of 50 mM imidazole/5 mM cysteine/1 mM $CaCl_2$ (pH 7.4) with or without the erythrosol fraction. After incubation, all membranes were washed twice with the isotonic buffer to remove cytosolic proteins adsorbed onto the membranes and then with 5 mM sodium phosphate buffer (pH 7.4) before final solubilization in 2% SDS. The membrane-free erythrosol fraction used was a concentrated preparation containing 14 g/100 ml hemoglobin and was dialyzed against 5 mM sodium phosphate buffer (pH 7.4) before use. This preparation had demonstrable calpain activity as assayed by the method of Murakami et al. [6] using casein as substrate. In the incubation medium of 0.8 ml, the erythrosol preparation used contained 0.1 unit of calpain. One unit of calpain is defined as the amount of the proteinase preparation catalyzing an increase of 1.0 absorbance unit at 750 nm.

In some experiments, membranes (350–400 μg protein) were incubated directly with preparations of Ca^{2+}-dependent proteinase and Ca^{2+}. The Ca^{2+}-dependent thiol proteinase was prepared from the erythrosol fraction by the method of Murakami et al. [6]. This preparation was substantially free of calpastatin and was sensitive to leupeptin. For incubation, membranes (350–400 μg protein) were treated with the proteinase preparation (0.15 calpain unit) for 30 min at 30°C in a medium containing 50 mM imidazole/5 mM cysteine/0.4 mM free Ca^{2+} (pH 7.4). Concentration of free Ca^{2+} was determined with a Ca^{2+} electrode (Radiometer, Copenhagen, Denmark).

Since human erythrocytes also contain aminopeptidase P which can be activated by divalent cations including Ca^{2+} [12], membranes (200 μg protein) were also digested in a separate experiment with aminopeptidase P preparation (0.48 units) at 37°C for 1 h in the presence of 25 mM Tris/0.11 mM $MnCl_2$ (pH 7.3). Mn^{2+} was used instead of Ca^{2+} because the enzyme is activated more by Mn^{2+} [12]. The aminopeptidase P preparation was a partially purified preparation that had been subjected to ethanol-chloroform extraction to remove hemoglobin and gel-filtration to remove contaminating proteins after the manner described by Sidorowicz et al. [12].

SDS-polyacrylamide gel electrophoresis. Membranes subjected to electrophoresis were boiled for 3 min with 2% SDS/15 mM dithiothreitol before the solubilized proteins were separated on a gradient slab gel (5–15% acrylamide) with the discontinuous system of Laemmli [13].

Immunoblot technique. Membrane proteins separated on SDS-polyacrylamide gels were allowed to react by immunoblot technique with a mono-

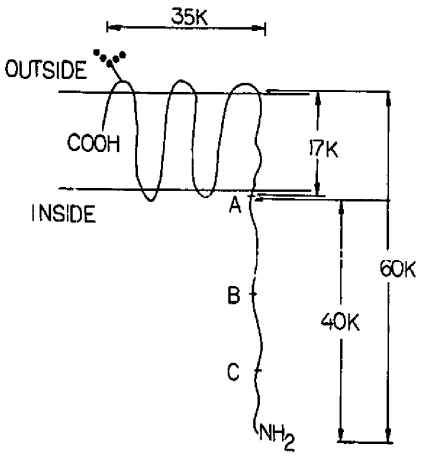

Fig. 1. Schematic diagram of band 3 and its degradation products in relation to the red cell membrane bilayer. Antisera used in the present study were raised against the 60 kDa and 52 kDa (35 kDa + 17 kDa) fragments [9]. Since the anti-60 kDa antibody binds to the cytoplasmic domain of band 3 close to the amino terminus, lower molecular weight fragments related to the 60 kDa peptide and located along the cytoplasmic domain can also react with the anti-60 kDa antibody. A, B and C are the proposed sites of attack by Ca^{2+}-dependent proteinase with the formation of 52 kDa, 70 kDa and 30 kDa fragments respectively.

specific polyclonal rabbit serum prepared against the purified 60 kDa fragment of the amino terminal portion of band 3 (Fig. 1). This antiserum also reacts with lower molecular weight fragments related to the 60 kDa peptide [9]. In some experiments, an antibody raised against the 52 kDa (35 kDa + 17 kDa) transmembrane carboxyl terminal segment of band 3 was used.

For immunoblotting, separated membrane proteins were first transferred to nitrocellulose paper [14,15]. After overnight running at 0.1 A, the nitrocellulose paper was removed, rinsed for 10 min with the isotonic phosphate-buffered saline, then for another hour with 20 mM Tris-HCl (pH 7.4) containing 0.02% NaN_3 and 2% casein to prevent nonspecific antibody binding before being incubated overnight with specific rabbit antibody diluted in the casein buffer. Unbound antibody was removed by washing five times (30 min each) with the casein buffer while bound antibody was detected by incubating the blot with ^{125}I-labeled protein A ($1 \cdot 10^6$ cpm/ml) for 1 h. This was followed by washing the blot seven times (15 min each) more with the casein buffer, drying on vacuum and finally autoradiography of the dried nitrocellulose blot with Kodak X-Omat AR film.

Results

When open membranes (400 μg protein/ml) derived from blood samples from which white cells had been completely removed were incubated at 37°C with 1 mM Ca^{2+} in the presence of 5 mM cysteine, no digestion of band 3 was observed even when incubation was continued up to 3 h or when 7.5 μM ionophore A23187 was included to improve Ca^{2+} accessibility to the membranes. If the erythrosol fraction (with demonstrable calpain activity) was included during the 3 h of incubation, however, degradation of band 3 occurred with generation of a 30 kDa fragment detectable by using the antibody raised against the 60 kDa amino terminal portion of band 3 (Fig. 2A). Formation of the 30 kDa fragment was found to be related to the action of a Ca^{2+}-dependent proteinase derived from the erythrosol fraction (Fig. 2C). Smaller 10 kDa fragments were also generated as a result of action of the Ca^{2+}-dependent proteinase preparation and Ca^{2+} on membrane-bound band 3 (Fig. 2C). Erythrosolic aminopeptidase P, on the other hand, was not involved in the generation of the 30 kDa and 10 kDa fragments (Fig. 2B). The very small amount of 30 kDa fragment that was formed might result from Mn^{2+} activation of residual Ca^{2+}-dependent proteinase present in the aminopeptidase preparation.

The 30 kDa fragment was derived from the amino terminal portion of band 3 since anti-60 kDa antibody was used for detection of this fragment. This was also concluded from a study employing membranes from a donor heterozygous for a variant of band 3 [10]. This variant has an elongated amino terminal portion for band 3 so that the 60 kDa, 40 kDa and 20 kDa fragments derived from this modified amino terminal portion appeared as doublets [9]. It was found that a 30 kDa doublet was also generated as a result of action of the Ca^{2+}-dependent proteinase preparation and Ca^{2+} on membrane-bound band 3 of the variant (Fig. 2D).

A quantitative analysis performed on band 3 fragments generated by digestion of the mem-

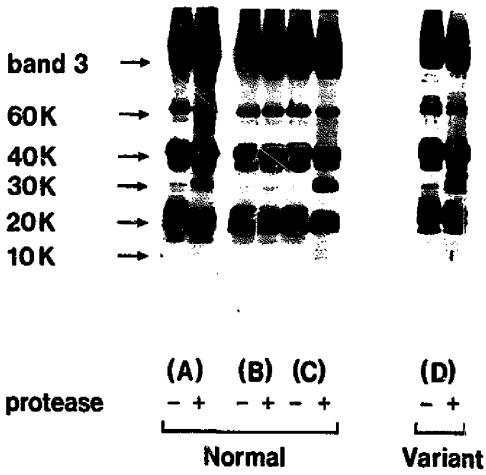

Fig. 2. Digestion of erythrocyte membranes of normal and heterozygote for the elongated variant of band 3 with (A) 1 mM $CaCl_2$ and erythrosol fraction, (B) 0.11 mM $MnCl_2$ and aminopeptidase P preparation isolated from erythrosol, and (C)/(D) 0.4 mM free Ca^{2+} and Ca^{2+}-dependent proteinase preparation isolated from the erythrosol fraction. (+) membranes incubated with proteinase preparation and divalent cation; (−) control membranes incubated with divalent cation alone. Digested membranes and their controls (10 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose paper and visualized by autoradiography using an antibody raised against the 60 kDa amino terminal portion of band 3 and ^{125}I-labeled protein A as described in Methods.

branes with the Ca^{2+}-dependent proteinase preparation further revealed a decrease in the amount of band 3 as well as that of its 60 kDa and 20 kDa fragments. The decrease was accompanied by a corresponding increase in the 40 kDa, 30 kDa and 10 kDa fragments (Table I), thus suggesting that the latter group of smaller fragments were indeed derived from band 3 and its bigger 60 kDa and 20 kDa fragments.

An antibody raised against the 52 kDa transmembrane carboxyl terminal portion of band 3 was also employed for following band 3 catabolism by the Ca^{2+}-dependent proteinase preparation. It was found that in the presence of 0.4 mM free Ca^{2+}, this proteinase increased the production of the transmembrane 52 kDa fragment and a 70 kDa fragment (Fig. 3A). Ca^{2+} by itself, however, was without effect. Based on the size of the

TABLE I

A QUANTITATIVE ANALYSIS OF BAND 3 FRAGMENTS AFTER DIGESTION OF MEMBRANES WITH Ca^{2+}-DEPENDENT PROTEINASE ISOLATED FROM THE ERYTHROSOL FRACTION

Autoradiographs obtained from control and proteinase-digested membranes, electrophoresed as described in Materials and Methods, were compared by densitometry. Anti-60 kDa antibody was employed for visualization. The total loss in intensity of band 3, 60 kDa and 20 kDa fragments (173244 + 22467 + 4746 = 200457) could account for the total intensity of the 40 kDa, 30 kDa and 10 kDa fragments (138445 + 49522 + 20615 = 208582). Increase (+); decrease (−).

Peptides	Band intensity after proteinase digestion (arbitary units)	% change
Band 3	−173244	−39.2
60 kDa	−22467	−38.6
40 kDa	138445	+33.4
30 kDa	49522	+1100
20 kDa	−4746	−3.4
10 kDa	20615	new appearance

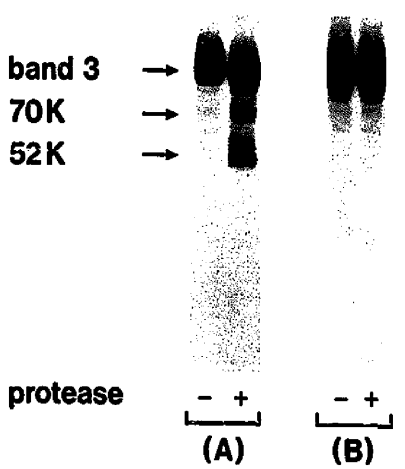

Fig. 3. Digestion of erythrocyte membranes with (A) 0.4 mM free Ca^{2+} and Ca^{2+}-dependent proteinase isolated from the erythrosol fraction, (B) 0.11 mM $MnCl_2$ and aminopeptidase P preparation isolated from erythrosol. (+) membranes incubated with proteinase preparation and divalent cation; (−) control membranes incubated with divalent cation alone. Digested membranes and their controls (10 μg protein) were subjected to electrophoresis, then transferred to nitrocellulose paper and visualized by autoradiography using an antibody raised against the 52 kDa transmembrane carboxyl terminal portion of band 3 and ^{125}I-labeled protein A as described in Materials and Methods.

generated fragments, it can be said that cleavages of band 3 occurred at the cytoplasm/membrane interface to generate the 52 kDa fragment and at another site 18 kDa from the interface on the cytoplasmic domain of band 3 to generate the 70 kDa fragment. The aminopeptidase P preparation, on the other hand, did not generate any fragment detectable by this antibody (Fig. 3B).

Discussion

In the present study, we show that Ca^{2+} mediates degradation of band 3 protein into discrete fragments by promoting the action of Ca^{2+}-dependent proteinase present in the erythrosol. Such a proteolytic event presumably only occurs when the red cell experiences a prolonged and uncontrolled increase of intracellular Ca^{2+} during certain pathological conditions or some physiological conditions like aging. Furthermore, owing to the coexistence of calpastatin in erythrosol, the action of the Ca^{2+}-dependent proteinase is expected to be manifested only if the level of calpastatin is low. Though such a condition has not yet been described in human, Pontremoli et al. [16] did report a defective calpain-calpastatin system in red cells from Milan hypertensive rats in which calpain level is normal but calpastatin concentration is 10-times below the normal level.

The observed cleavage of band 3 at the cytoplasm/membrane interface and along the cytoplasmic domain of the protein is what is expected of the action of an intracellular proteinase. No degradation of band 3 at the external surface of the red cell membrane due to the erythrosolic Ca^{2+}-dependent proteinase was observed in spite of the use of open membranes. This suggests that the proteinase can recognize specific sites along band 3 protein. The presence of the membrane lipid bilayer probably helps to ensure specificity of the proteinase action by providing protection to the transmembrane portion of band 3 against attack by the Ca^{2+}-dependent proteinase. This is evident from the failure of identification of a transmembrane carboxyl terminal fragment of band 3 smaller than 52 kDa after digestion of membranes with the proteinase and the finding that only the cytoplasmic domain of membrane-bound band 3 is degraded to give 30 kDa and 10 kDa fragments.

The 30 kDa and 10 kDa fragments of band 3, formed as a result of the action of erythrosolic Ca^{2+}-dependent proteinase, were not reported in an earlier study by Morrison et al. [9] though the same antibody which recognizes the 60 kDa amino terminal portion of band 3 was used for identification of band 3 fragments (60 kDa, 40 kDa and 20 kDa) isolated from circulating erythrocytes. Most probably, this is because those cells from which these band 3 fragments were isolated had never experienced a drastic increase in Ca^{2+} level big enough to activate intracellular Ca^{2+}-dependent proteinase, while cells, if any, that had high Ca^{2+} levels would have been eliminated already and were thus not harvested from the circulating erythrocyte population for study.

It is interesting to note that the transmembrane carboxyl terminal 52 kDa fragment generated as a result of the action of Ca^{2+}-dependent proteinase contains components that make up the senescent cell antigen, which is located on an extracellular portion of band 3 that includes most of the carboxyl terminal segment and 30% of the 17 kDa anion-transport region [17]. The portion of band 3 containing the senescent cell antigen might be derived from further processing of the 52 kDa transmembrane fragment. Furthermore, in cells with high intracellular Ca^{2+} concentration, activated transglutaminase might act on the generated fragments to form protein aggregates hence resulting in loss of membrane deformability.

The role played by the cytoplasmic domain of band 3 in interacting with cytosolic and cytoskeletal components might be affected as a result of cleavages occurring along its length. However, since those fragments generated from the cytoplasmic domain are not detached from the membrane, some of these functions of band 3 are not affected. The binding of glyceraldehyde-3-phosphate dehydrogenase to the membrane via band 3, for example, is not affected as a result of digestion of the membranes with the Ca^{2+}-dependent proteinase (Au, K.S., unpublished data). Whether the interaction of band 3 with ankyrin, which involves regions of the cytoplasmic domain closer to the lipid bilayer and specific conformation of the cy-

toplasmic domain [4], is affected by the Ca^{2+}-mediated catabolism of band 3 remains to be seen.

References

1 Childs, R.A., Feizi, T., Fukuda, M. and Hakomori, S.I. (1978) Biochem. J. 173, 333–336.
2 Krusius, T., Finne, J. and Rauvala, H. (1978) Eur. J. Biochem. 92, 289–300.
3 Kay, M.M.B., Goodman, S.R., Sorenson, K., Whitfield, C.F., Wong, P., Zaki, L. and Rudloff, V. (1983) Proc. Natl. Acad. Sci. USA 80, 1631–1635.
4 Low, P.S. (1986) Biochim. Biophys. Acta 864, 145–167.
5 Lorand, L., Bjerrum, O.J., Hawkins, M., Lowe-Krentz, L. and Siefring, G.E., Jr. (1983) J. Biol. Chem. 258, 5300–5305.
6 Murakami, T., Hatanaka, M. and Murachi, T. (1981) J. Biochem. 90, 1809–1816.
7 Hatanaka, M., Kikuchi, T. and Murachi, T. (1983) Biomed. Res. 4, 381–388.
8 Lorand, L. and Michalska, M. (1985) Blood 65, 1025–1027.
9 Morrison, M., Grant, W., Smith, H.T., Mueller, T.J. and Hsu, L. (1985) Biochemistry 24, 6311–6315.
10 Mueller, T.J. and Morrison, M. (1977) J. Biol. Chem. 252, 6573–6576.
11 Beutler, E., West, C. and Blume, K.G. (1976) J. Lab. Clin. Med. 88, 328–333.
12 Sidorowicz, W., Canizaro, P.C. and Bevhal, F.J. (1984) Am. J. Hematol. 17, 383–391.
13 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
14 Gershoni, J.M. and Palade, G.E. (1983) Anal. Biochem. 131, 1–15.
15 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
16 Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. and Bianchi, G. (1986) Biochem. Biophys. Res. Commun. 139, 341–347.
17 Kay, M.M.B. (1984) Proc. Natl. Acad. Sci. USA 81, 5753–5757.