Degradation of Transmembrane Proteins in Ca\textsuperscript{2+}-enriched Human Erythrocytes

AN IMMUNOCHEMICAL STUDY*

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APART FROM CAUSING THE FORMATION OF \(\gamma\)-glutamyl-\(\epsilon\)-lysine cross-linked polymers, exposure of human erythrocytes to Ca\textsuperscript{2+} and ionophore A23187 leads to a breakdown of the two major transmembrane proteins, i.e., the anion-transporting band 3 and glycophorin. This apparently proteolytic phenomenon was examined by crossed immunoelectrophoretic techniques. The main product of the cleavage of band 3 had a chain weight of about 55,000 and showed good precipitation with the antibody raised against the intact protein. The degradation of glycophorin was more rapid and, when complete, gave rise to small fragments which were barely precipitated with anticytrophorin antibody. Incubation of the cells with pepstatin or N-ethylmaleimide prior to and during Ca\textsuperscript{2+} loading prevented the breakdown of both transmembrane proteins. Histamine, a competitive inhibitor of the transglutaminase-catalyzed formation of \(\gamma\)-glutamyl-\(\epsilon\)-lysine cross-links in Ca\textsuperscript{2+}-enriched erythrocytes, also provided some protection, suggesting that the breakdown of the two transmembrane proteins might be in some manner related to the transglutaminase-dependent polymerization process. Pathophysiological implications of the proteolytic event, which would distort the normal interaction of membrane proteins with the cytoskeleton, are discussed.

Previous work from this laboratory with Ca\textsuperscript{2+}-loaded human erythrocytes focused on the fusion of membrane proteins by \(\gamma\)-glutamyl-\(\epsilon\)-lysine bonds (1-8). A variety of evidence, such as changes in the electrophoretic profile of ghost proteins isolated from cells following enrichment with Ca\textsuperscript{2+} in the presence or absence of amines, Ca\textsuperscript{2+}-dependent incorporation of labeled amines into membrane proteins in intact red cells, and a direct immunoelectrophoretic analysis of the polymer, already showed that formation of the \(\gamma\)-glutamyl-\(\epsilon\)-lysine cross-linked membrane clusters involved spectrin, band 3, 2.1 (9-18), and the 4.1 protein.

In the course of studying membrane protein alterations during exposure of human red cells to Ca\textsuperscript{2+} and ionophore A23187, we have noticed yet another type of reaction, possibly proteolytic in nature, which affects membrane proteins and which is clearly distinguishable from the transglutaminase-catalyzed event. It is characterized by degradation of the two main transmembrane proteins, band 3 and glycophorin. 1

MATERIALS AND METHODS

Erythrocytes were obtained by centrifuging fresh citrated blood (3500 rpm x 5 min) and by washing four times with a buffer comprising 100 mM KCl, 60 mM NaCl, 10 mM glucose, and 5 mM Tris-HCl (pH 7.4). The cells were suspended in this buffer to hematocrit values between 20 and 50%.

Ionophore A23187 was purchased from Calbiochem-Behring, N-ethylmaleimide from Eastman, and histamine dihydrochloride from Sigma. Pepstatin, leupeptin, and antipain were obtained through the courtesy of the United States-Japan Cooperative Cancer Research Program. Stock solutions of the ionophore (5 mM) and of pepstatin (200 mM) were prepared in dimethyl sulfoxide. N-ethylmaleimide was made up fresh each time to a concentration of 500 mM in the buffer used for suspending erythrocytes. The histamine salt was dissolved (500 mM) in 100 mM KCl, 10 mM glucose, and 5 mM Tris-HCl and was neutralized with NaOH. Stock solutions of leupeptin (200 mM) and of antipain (100 mM) were prepared in 10 and 50 mM Tris-HCl (pH 7.4), respectively.

Loading of the red cells with Ca\textsuperscript{2+} (1.5 and 2 mM) was carried out at 37 °C in the presence of the ionophore (10 and 20 mM) for the periods specified. Pretreatments with pepstatin, leupeptin, antipain (1-2 mM), N-ethylmaleimide (5 mM), or histamine (60 mM) were for durations of 30 min at 37 °C; the concentration of dimethyl sulfoxide in the incubation mixtures varied between 0.4 and 1%. Appropriate controls were run with equivalent dimethyl sulfoxide. In order to guard against possible proteolytic degradation during the preparation of ghosts, in most experiments 1 mM benzamidine (Sigma), 1 mM fresh phenylmethanesulfonyl fluoride (Sigma), and 0.5 mM iodoacetamide (Sigma) were included in the 8 mM phosphate buffer (pH 8) used for lysing of cells.

Isolation of erythrocyte ghosts and the electrophoretic analysis in 4.5% polyacrylamide in sodium dodecyl sulfate under reduced conditions were carried out according to published procedures in tube or slab gels (9, 10). The Coomassie blue-stained disc gels were scanned in a Gilford linear transport system at 540 nm.

Combined sodium dodecyl sulfate (Aldrich)-polyacrylamide (5%; Bio-Rad) and -agarose (1%, Marine Colloids, type LE) crossed immunoelectrophoresis was carried out as described by Bjerrum and Bhakdi (11). Following solubilization in 2% SDS and 40 mM dithio-

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proteins were subjected to SDS-PAGE in 1.5-mm thick slab gels using threitol (15 min, 37 °C), 20-40-μg samples of solubilized membrane proteins were subjected to SDS-PAGE in 1.5-mm thick slab gels using the procedure of Fairbanks et al. (9) with 0.1% SDS, and the runs were terminated when the pyronin-tracking dye migrated to a distance of 7.5 cm. The polyacrylamide support was trimmed to a width of 0.75 cm and was washed for 15 min at 22 °C in a buffer containing 38 mM Tris, 100 mM glycine (pH 8.7), and 1% Lubrol PX (Sigma). The slice was then placed 2 cm above the cathodic edge of the plate (7 × 10 cm) used for crossed immunoelectrophoresis, on top of a 2-mm thick and 4-cm wide agarose layer, which contained 3.5% Lubrol PX. The two layers were then sealed by application of a few drops of warm agarose (with 3.5% Lubrol PX) at the cathodic side. The antibody-containing agarose with 2% Lubrol PX was cast as a 6-cm wide and 1.5-mm thick layer on the anodic side of the plate. Electrophoresis was carried out at 2 V/cm for about 16 h, using the Tris glycine buffer of pH 8.7 without Lubrol PX. The polyacrylamide band was removed prior to pressing, washing, and staining of the agarose.

Rabbit antibody against human erythrocyte ghost (see Refs. 11 and 12) was obtained from Dako Corp., Santa Barbara, CA (A 104, lot 099A). To 6 ml of antibody solution, 50 μl of human serum albumin (Pentex; 5 mg of lyophilized protein/ml of 100 mM NaCl) were added. Following incubation at 4 °C overnight, the precipitate was removed by centrifugation at 50,000 × g for 15 min. Antiglycophorin antibody was raised in rabbits against a glycophorin preparation obtained by the procedure of Marchesi and Hargsd (13). Immunization and isolation of the antibody were carried out as recommended by Harboe and Ingild (14). Specificity of the antiseraum against glycophorin was assayed by absorption with EDTA-extractable erythrocyte membrane proteins and with a small amount of washed ghosts, not sufficient to cause an appreciable reduction in antglycophorin titer. Anti-band 3 protein antibody was obtained by immunizing rabbits against the proteins eluted from the band 3 regions following SDS electrophoresis of erythrocyte ghosts on polyacrylamide gels (15). The purified antibody, which had an equally high titer against band 3 and glycophorin, was sequentially absorbed with whole erythrocytes (16) and then with a small amount of purified glycophorin and with EDTA-extractable membrane proteins. The efficacy of the absorption procedures was checked throughout by crossed immunoelectrophoresis, with antie-rythrocyte membrane antibody in the upper reference gel (17). The anti-band 3 and the antiglycophorin antibodies (prepared at The Protein Laboratory of the University of Copenhagen) were dissolved in 0.1 M NaCl containing 15 mM NaN₃, with aprotinin (Bayer, Germany) added per ml of solution.

RESULTS

Degradation of the band 3 protein in membranes of Ca²⁺-loaded human erythrocytes may be readily recognized on the protein profile of ghosts using SDS-PAGE procedures (Fig. 1). Following extended periods of incubation, the amount of band 3 which disappears often seems to be out of proportion with the amount of γ-glutamyl-ε-lysine cross-linked polymer formed (Fig. 1, right, X) and, more importantly, a rather strongly staining band, corresponding to a polypeptide with a weight of 55,000, appears in the 4.5 region of the gel. Pepstatin (18) can prevent the degradation of band 3 (Fig. 2). Fig. 2 represents a semiquantitative attempt to illustrate the protective effect of 1 mM pepstatin which was added to the medium prior to and during the loading of erythrocytes with Ca²⁺ (1.5 mM) in the presence of ionophore A23187 (10 μM). Peaks corresponding to the band 3 and 4.5 regions in the gel scans were cut out and weighed, and the percentage of band 3 remaining was computed according to the formula given in the legend of Fig. 2.

Crossed immunoelectrophoresis following the SDS-PAGE separation of membrane proteins in the first dimension revealed that, in accord with our earlier observation (7), glycophorin underwent an even more dramatic degradation than band 3 in Ca²⁺-loaded erythrocytes. Using a polyspecific rabbit antibody raised against the human ghosts, patterns such as shown in Fig. 3 (B) were obtained for cells treated with Ca²⁺ for 12 h. The main degradation product of band 3 (marked as 3') corresponds to the fragment with an approximate chain weight of 55,000 which appears as band 4.5 in one-dimensional SDS-PAGE, and it shows immunological identity with the band 3 protein. Almost no 3' peak is seen in the Mg²⁺ controls (Fig. 3, A) or in Ca²⁺ incubation experiments of short duration (<10 min). However, with longer times, as the band 3 peak decreases, there is an increase in 3'. Glycophorin, which represents one of the strongest antigens among membrane proteins, gives the darkest and best defined precipitation line in crossed immunoelectrophoresis (see Fig. 3, A, GP). Nevertheless, after 12 h of incubating the cells in the Ca²⁺-containing medium with ionophore, practically no glycophorin remained (see Fig. 3, B). Significant degradation of glycophorin is seen within a few minutes (10-20 min) after the exposure of the cells to Ca²⁺ and, by a few hours (>2 h), almost no precipitable antigen remains. The relative intactness of the peaks for band 3 and for glycophorin in the Mg²⁺ controls (Fig. 3, A) proves that the breakdown of both proteins is, indeed, a Ca²⁺-specific phenomenon and that it is not due in any way to the presence of either ionophore or dimethyl sulfoxide in the medium. This

![Fig. 1. Degradation of band 3 membrane protein in Ca²⁺-enriched human erythrocytes. Reduced protein profiles in SDS-PAGE (9) are shown for ghosts obtained from cells which were exposed (at 37 °C for 18 h) in the presence of 10 μM ionophore A23187 to either 1.5 mM MgCl₂ (left, control) or to 1.5 mM CaCl₂ (right, gel). The cells (50% hematocrit) were incubated in a solution of 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, and 5 mM Tris-HCl (pH 7.4). Anodic side is at the bottom. Designations of polypeptide components of interest are given by numbers 1-4.5; the γ-glutamyl-ε-lysine cross-linked polymer is denoted by X on top of the gel.](http://www.jbc.org/)

![Fig. 2. Pepstatin inhibits the breakdown of the band 3 membrane protein. In the experiments marked by circles (○), the protocol was similar to the one employed in Fig. 1 for the right-hand gel, whereas in the experiments marked by triangles (△), 1 mM pepstatin was also present during incubation of the red blood cells (RBC) for the periods shown on the abscissa. The percentage of band 3 remaining (ordinate) was computed from gel scans by measuring the area of the protein peak at the position of band 3, multiplying it by 100, and dividing it by the combined area under bands 3 plus 4.5 (i.e. per cent of band 3 remaining = (band 3 peak/band 3 peak + band 4.5 peak) × 100).](http://www.jbc.org/)
FIG. 3. The breakdown of the band 3 protein and glycophorin in Ca$^{2+}$-loaded erythrocytes can be prevented by the addition of pepstatin or N-ethylmaleimide, as shown by crossed immunoelectrophoretic analysis using antibody against whole erythrocyte membrane. SDS-PAGE in the first dimension on 25 μg of membrane proteins (with arrows pointing in the anodic direction) was followed by immunoelectrophoresis in the second dimension (anode on top), against an antimembrane antibody (10 μl/cm²), as described under "Materials and Methods." For the experiment shown in A, the cells were incubated over a period of 12 h in 20 μM ionophore with 1.5 mM MgCl$_2$ (control); for B, with 1.5 mM CaCl$_2$; for C, with CaCl$_2$ and 2 mM pepstatin; and for D, with CaCl$_2$ and 5 mM N-ethylmaleimide. The band 3 protein and its major degradation product are designated as 3 and 3', respectively. The angled solid lines point to the glycophorin (GP)-related immunoprecipitate.

is further underlined by the results of experiments in which the concentration of Ca$^{2+}$ in the medium was varied in the presence of ionophore; in 12 h of incubation, appreciable breakdown of glycophorin and band 3 was observed only if the Ca$^{2+}$ concentration was raised above 0.2 mM. No degradation of the transmembrane proteins occurred when the cells were incubated in 1.5 mM CaCl$_2$ in the absence of the ionophore.

The crossed immunoelectrophoretic approach seemed to be well suited for the examination of inhibitors which could be used to prevent the breakdown of the two transmembrane proteins. Fig. 3, C, pertaining to an experiment of 12 h of Ca$^{2+}$ loading, shows that pepstatin can prevent not only the degradation of band 3 but that of glycophorin as well. N-Ethylmaleimide was similarly effective in inhibiting the breakdown of both transmembrane proteins (Fig. 3, D).

These findings were confirmed separately with monospecific rabbit antibodies raised either against the human band 3 protein or against human glycophorin. Illustrations are given in Figs. 4 and 5 in relationship to the breakdown of the two proteins in erythrocytes exposed to Ca$^{2+}$ and ionophore for periods of 12 h in the presence and absence of 1 mM pepstatin. The findings in Fig. 4 are of additional interest because they draw attention explicitly to the existence of band 3-related fragments of chain weights much lower than the 55,000 species designated as 3'. In fact, immunoprecipitates of small fragments moving close to the tracking dye are readily detectable in Fig. 4.

Some additional aspects of the inhibition by pepstatin are noteworthy. First, its effect is specific in the sense that neither leupeptin nor antipain, two other protease inhibitors of microbial origin (18), could match the potency of pepstatin. Second, in contrast to N-ethylmaleimide which protects Ca$^{2+}$-loaded cells against the formation of γ-glutamyl-ε-lysine cross-linked polymers as well as against the breakdown of the transmembrane proteins, the effect of pepstatin appears to be rather selective in that the transglutaminase-dependent formation of polymeric material can proceed even in the presence of pepstatin.

Attempts for achieving the converse, i.e. of inhibiting the transglutaminase-catalyzed polymerization event without affecting the degradation of the band 3 protein and/or glycophorin, have not met with success thus far. Crossed immunoelectrophoretic examination of membranes from cells exposed to Ca$^{2+}$ and ionophore for a period of 3 h in the presence of 60 mM histamine showed that the latter compound which is a known competitive inhibitor of the transglutaminase-mediated cross-linking reaction in erythrocytes provided significant protection for preserving both glycophorin and the band 3 proteins.³

³ Experiments utilizing lower concentrations of histamine over longer time periods led us to suggest earlier that the degradation of the two transmembrane proteins may not have been affected by amines.
FIG. 4. Inhibition of band 3 breakdown by pepstatin, as demonstrated by crossed immunoelectrophoresis with a monospecific antibody against the band 3 protein. The experimental design corresponds to that given for Fig. 3 except that 30 µg of membrane proteins, 1 mm pepstatin, and monospecific anti-band 3 antibody (18 µl/cm²) were used. For the experiment in A, the cells were exposed for 12 h to CaCl₂, in the presence of pepstatin; and for B, only to CaCl₂.

FIG. 5. Inhibition of glycophorin breakdown by pepstatin, as shown by crossed immunoelectrophoresis with a monospecific antibody against glycophorin. The design of this experiment corresponds to that in Fig. 4 except that a glycophorin-specific antibody (4 µl/cm²) was used. For A, the cells were exposed to CaCl₂ (12 h) in the presence of pepstatin; and for B, only to CaCl₂. GP-1 and 2 denote the normal glycophorin bands; GP' represents small, immunoprecipitating fragments from these proteins.

DISCUSSION

This paper presents an immunochemical analysis of the phenomena of band 3 and glycophorin degradation occurring in intact human erythrocytes upon an increase in the concentration of intracellular Ca²⁺ ions. Band 3 is the major transmembrane protein of the red cell (see Ref. 19) responsible for anion transport; it interacts with ankyrin (i.e., band 2.1) which serves as a bridge to spectrin and thus to the cytoskeletal reticulum (see Ref. 20). In addition, the cytoplasmic domain of band 3 is known to bind to hemoglobin (21), glyceraldehyde-3-phosphate dehydrogenase (22), aldolase (23), and phosphofructokinase (24) in a specific manner. Glycophorin, another important transmembrane protein (13), carries the M and N blood group antigens (25), and it also seems to be involved in the reactions of the erythrocyte with other cells and organisms, such as the malaria parasite (26). Linkage of glycophorin to the cytoskeleton via band 4.1 has recently been suggested (27).

Degradation of band 3 protein has frequently been observed in ghost preparations regardless of whether they were stored in the absence (28) or presence (29, 30) of Ca²⁺ or whether they were extracted with detergents (31). These reports cannot be readily compared with the phenomenon described by us. The only other instance where a decrease of band 3 with the concomitant appearance of band 4.5 was noted was in intact human erythrocytes during incubation (at 37 °C) in Krebs-Ringer solution which, incidentally, contained Ca²⁺. Sialic acid release began at 9 h and reached its highest value (50-70% of the total membrane sialic acid) after 25-30 h of incubation when a change in the PAS 1 to PAS 2 ratio became apparent.

As shown in this paper, immunochemical evaluation of band 3 and glycophorin breakdown has many advantages over more conventional analytical approaches. In the case of Ca²⁺-loaded erythrocytes, for example, a finding of reduction in staining intensity or disappearance of a normal band on the reduced SDS-PAGE membrane protein profile could mean proteolysis (i.e., displacement towards smaller molecular weight components) or cross-linking by transglutaminase (i.e., displacement upward towards larger components). By the same reasoning, the appearance of a novel band could be interpreted either in terms of the proteolytic breakdown of larger proteins or the polymerization of smaller units. In the absence of independent means of identification, any conclusion as to the fate of a given protein component would be tentative at best. Immunological identity (see Figs. 3-5) is an important marker in appraising alterations of protein structures in the membrane of the erythrocyte following various treatments.

Our data show rather conclusively that, upon Ca²⁺ loading, there is a progressive breakdown of band 3 and glycophorin in the membranes of intact human erythrocytes. A possible interpretation is that the increase of intracellular Ca²⁺ causes activation of a protease responsible for degrading these transmembrane proteins. Ca²⁺-activated thiol proteases of many cells have been studied extensively in recent years (e.g., in muscle (35) and in platelets (36, 37)). Inhibition by N-

Although the phenomenon seems to be quite general, the erythrocytes of one (healthy) donor showed an unusual susceptibility for the breakdown of transmembrane proteins upon Ca²⁺ loading.
ethylnleimide (see Fig. 3, D) might be explained by the participation of a thiol protease in the observed degradation of band 3 and glycoporin. On the other hand, it is also possible that Ca$^{2+}$ causes some change in the membrane by affecting the phospholipid bilayer, by causing an oxidative (38, 39) or transglutaminase-dependent (2, 3) cross-linking of proteins with concomitant alteration of shape and viscoelastic properties, so that the transmembrane proteins become more susceptible to the actions of intracellular proteolytic enzymes in general. A number of proteases have been recognized and have, indeed, been isolated from red cells (40-45). The inhibition of band 3 and glycoporin degradation by pepstatin (see Figs. 2-5) might indicate that acidic endopeptidases associated with the erythrocyte membrane are involved. Three different enzymes of this type have been purified from the ghosts of human red cells and all of them were shown to be sensitive to inhibition by pepstatin (47, 48). Nevertheless, when dealing with intact erythrocytes, it must also be borne in mind that pepstatin could have an effect totally unrelated to its forming tight complexes with acid proteases. Pepstatin, for example, binds to the surface of human neutrophils in a rather specific manner and, because it shares binding sites with formylmethionylleucylphenylalanine, it acts as a chemo-attracting agent (49, 50).

Whatever the explanation for the observed effect of pepstatin might be, it is the only agent thus far which allows us to inhibit differentially the proteolytic degradation of band 3 and glycoporin without interfering with the transglutaminase-catalyzed production of $\gamma$-glutamyl-$\epsilon$-lysine cross-linked polymers in the membranes of Ca$^{2+}$-loaded erythrocytes. We have been unable to obtain the converse in a clear-cut manner, i.e. to suppress the cross-linking reaction without inhibiting protelysis at the same time. N-Ethylmaleimide inhibits both but, more surprisingly, so does histamine. The latter is not an inhibitor of transglutaminase but serves as an alternate substrate for the enzyme (3). When applied to intact cells, relatively large concentrations of the amine are required to inhibit the competitive cross-linking of membrane proteins, and the inhibiting effect is of relatively short duration. Nevertheless, unless it is assumed that histamine could act as an inhibitor of red cell proteases as well, the proteolytic degradation of the transmembrane proteins must be somehow coupled to the transglutaminase-catalyzed cross-linking event. As suggested before, distortion of membrane structure by cross-linking might perhaps enhance proteolysis. It is noteworthy that cross-linking and proteolysis have similar Ca$^{2+}$ concentration dependence.

The use of pepstatin, as a selective inhibitor of band 3 and glycoporin degradation without preventing the transglutaminase-catalyzed cross-linking, should greatly aid in evaluating the relative significance of these two apparently concurrent reactions on changes of the physical properties of the membrane in Ca$^{2+}$-loaded erythrocytes, such as loss of deformability (6), increased density (51, 52), and an irreversible fixation of shape change (3, 4, 6). Furthermore, the proteolytic phenomenon induced experimentally in the erythrocytes by treatment with Ca$^{2+}$ and ionophore may have relevance for the survivability of red cells stored in blood banks (63) and for a variety of pathophysiological situations in which rather high (approximately 0.2 mm) intracellular concentrations of Ca$^{2+}$ are known to exist (old red cell populations (54), sickle cells (55, 56), and energy-depleted cells (57)).

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