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THE EFFECT OF ENDOGENOUS PROTEASES ON THE SPECTRIN BINDING PROTEINS OF HUMAN ERYTHROCYTES

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Summary

We have demonstrated that in human erythrocyte ghosts endogenous proteolytic activity is responsible for the digestion of the spectrin binding proteins (bands 2.1 to 2.6). The pH optimum, cofactor requirements and inhibitor sensitivity have been established. Our results indicate that proteolysis of bands 2.1 to 2.6 and the formation of 3′, a fragment containing an active spectrin binding site, can occur through two enzymatic pathways: a cascade of consecutive proteolytic cleavages of the spectrin binding proteins inhibited by phenylmethylsulfonyl fluoride or a Ca^{2+}-stimulated, phenylmethylsulfonyl fluoride-insensitive, EDTA-inhibited cleavage of band 2.1 to band 2.3, followed by digestion to band 3′ by phenylmethylsulfonyl fluoride-inhibitable enzymes. These findings may provide the techniques necessary to prevent proteolysis of the spectrin binding proteins during purification and reconstitution experiments and provide insight into how they are formed in vivo.

Introduction

Spectrin binding proteins of the human erythrocyte membrane have recently been identified as band 2.1 * [1—3] and the family of proteins with similar peptide maps [1,3] which consists of bands 2.2 to 2.6 **. Limited treatment of

* Nomenclature for human erythrocyte polypeptides, according to Steck [23].

** Band 2.1 has been named 'ankyrin' [2], but this name has previously been used to describe another protein [24]. Band 2.1 and the series of polypeptides related to band 2.1 by sequence homology have been called 'syndeins' [3].

Abbreviations: SDS, sodium dodecyl sulfate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethyl sulfonyl fluoride; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; Me_2SO, dimethyl sulfoxide.

inside-out spectrin-depleted vesicles with α-chymotrypsin reduces their spectrin binding ability and releases a water-soluble 72 000 dalton fragment which associates with spectrin in solution and inhibits the binding of spectrin to undigested spectrin-depleted inverted vesicles [4,5]. Another fragment demonstrating spectrin binding activity is band 3′, a polypeptide which is produced with a concomitant loss of the band 2.1 family in ghost preparations that are incubated at 4°C [1,3]. This fragment migrates with band 3 in SDS-polyacrylamide gel electrophoresis and remains associated with the insoluble spectrin-actin residue left after Triton X-114 solubilization of intact erythrocytes [6,7]. The formation of 3′ with time has been attributed to an endogenous proteolytic event [3] and provides the focus for our present study.

Proteases endogenous to the red cell were first reported by Morrison and Neurath [8]. Two proteolytic activities with pH 3 and 8 optima were found in crude stroma suspensions using hemoglobin and casein as protein substrates. Tökes and Chambers [9] measured the release of radioactivity from ^{125}I-labelled casein covalently linked to latex beads and found proteolysis at pH 8 stimulated by the non-ionic detergent NP-40 and inhibited to varying degrees by diisopropylphosphorofluoridate, Zn^{2+}, TLCK, PMSF and EDTA. Triplett et al. [10] and Carraway et al. [11] reported Ca^{2+}-induced proteolysis of membrane proteins at physiological pH which generated a 174 000 dalton polypeptide and caused a diminution of a 91 000 dalton polypeptide. These effects were only observed when Ca^{2+} was present during erythrocyte hemolysis and could be prevented by chelating agents which were added prior to or simultaneously with the Ca^{2+}. Without adding Ca^{2+} during lysis, King and Morrison [12] saw similar proteolytic effects with isolated washed ghost membranes. Quirk et al. [13] studied proteolytic alterations in protein patterns derived from human erythrocytes fused by oleoylglycerol. Changes in the band 2.1 to 2.3 and band 6 regions were observed, bands 3 and 4.1 decreased, bands 4.3 and 4.5 increased and a new component in the band 3 region was formed. Recently, Allen and Cadman [14] claimed that cytosolic proteases, which appeared to adsorb to the membrane in the presence of Ca^{2+} and to be extractable with EDTA, caused a decrease in bands 1, 2, 3 and 4.1 and the appearance of multiple lower molecular weight bands.

The wide variations in experimental conditions employed in these studies have precluded a clear understanding of the nature of the proteolytic events in vitro and the physiological function they may serve in vivo. The present investigation focuses on the interaction of endogenous erythrocyte membrane proteases with the band 2.1 family of proteins. We show that two different proteolytic pathways can generate this family of proteins from band 2.1 in vitro and we determine the pH optima, inhibitor requirements and cofactors needed by these proteases for the conversion of band 2.1 to 3′, all under the same general conditions.

Experimental procedures

Materials. α-Chymotrypsin (EC 3.4.21.1; 50 units/mg), TLCK-treated α-chymotrypsin (50 units/mg), TPCK, PMSF, *N*-benzoyl-L-tyrosine ethyl ester, α-cellulose and microcrystalline cellulose (mean size 50 μm) were from Sigma.

All materials used for SDS-polyacrylamide gel electrophoresis were from Bio Rad Laboratories.

Methods. Erythrocytes from freshly-drawn human blood were washed three times in 15—20 vols. of 310 mosM sodium phosphate (pH 7.4) and incubated at a 50% hematocrit with either α-chymotrypsin or TLCK-treated α-chymotrypsin (200 μg/ml final concentration) for 1 h at 37°C. The cells were washed twice and brought back to 50% hematocrit, and the chymotrypsin reaction was inhibited by treatment with TPCK (100 μg/ml, 0.5% Me_2SO (v/v)) for 5 min at 37°C. After two additional washes in 310 mosM sodium phosphate (pH 7.6) erythrocyte ghosts were prepared by lysing the cells in 20 mosM sodium phosphate (pH 7.6) as described by Dodge et al. [15]. For some experiments, the lysis buffer contained a 1 mM concentration of TPCK, PMSF, EDTA, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, or $FeCl_2$. TPCK-inhibition of chymotrypsin in the presence and absence of ghosts was determined using *N*-benzoyl-L-tyrosine ethyl ester as substrate as described by Hummel [16]. The possibility that contaminating leukocytes or platelets might account for our results was ruled out by checking all of our experimental observations with ghosts derived from blood that had been passed through a column of microcrystalline cellulose and α-cellulose as described by Beutler et al. [17]. The preparation of spectrin-depleted inside-out vesicles was performed as previously described [4] and SDS-polyacrylamide gel electrophoresis was performed by the method of Fairbanks et al. [18] as modified by Steck and Yu [19]. The reader should note that resolving band 2.1 from spectrin in gel photographs is difficult when the gels are adequately loaded to reveal the other polypeptides in the band 2.1 region. The cleavage of band 2.1 is more easily monitored by observing the formation of its lower molecular weight breakdown products, particularly band 3′.

All experiments, except that of Fig. 6, were repeated at least twice. Within each experiment, with a single batch of blood, the results were always consistent although slight variations in enzyme activity were associated with different batches of blood. To control this, some cells in each experiment were lysed in the absence of any additional agents to establish a baseline level of proteolytic activity.

Results

Formation of 3′ is the result of endogenous proteases

To monitor the appearance of 3′, it is necessary to cleave band 3 before hemolysis by treating the intact erythrocytes with α-chymotrypsin from the outside and then inhibiting the reaction with TPCK, as described in Experimental procedures. Fig. 1A shows the formation of 3′ and the concomitant loss of the band 2.1 family of proteins in ghosts incubated at 4°C which are prepared from these cells. To test whether these effects are the result of residual chymotrypsin, cells were lysed and washed in the presence of 1 mM TPCK or PMSF. It is clear that TPCK, a specific inhibitor of chymotrypsin, has no effect on the observed proteolytic events (Fig. 1B), whereas equimolar concentrations of PMSF, a general serine-protease inhibitor, prevents band 2.1 digestion and the formation of 3′ (Fig. 1C). Using an assay for chymotrypsin activity similar to that of Hummel [16], we were able to show that under conditions identical

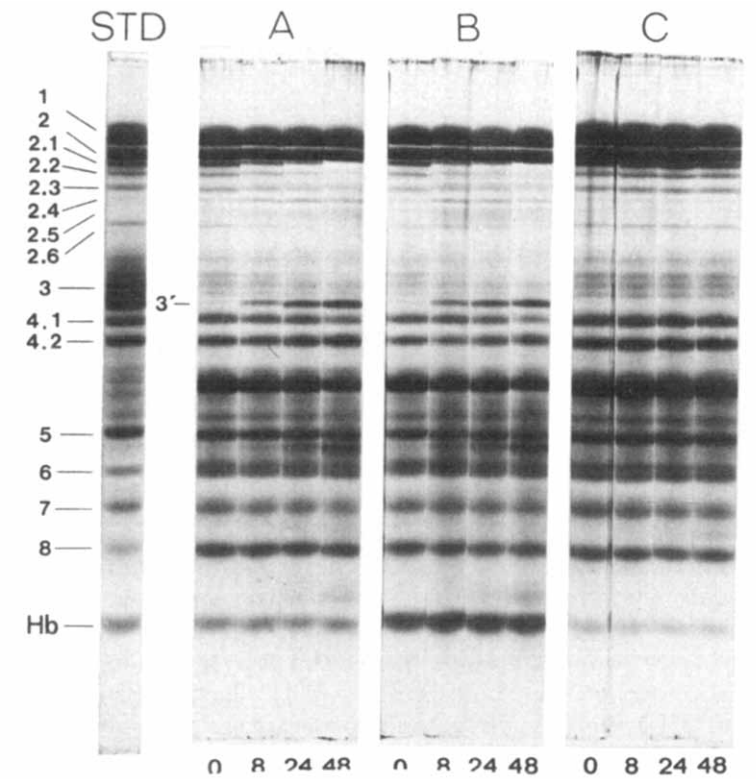


Fig. 1. Formation of 3′ in the presence of protease inhibitors. Erythrocytes pretreated with α-chymotrypsin were lysed and washed in 20 mosM sodium phosphate (pH 7.6) in the absence (A) or presence of 1 mM TPCK (B) or 1 mM PMSF (C). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. STD, ghost standard; Hb, hemoglobin; band labelling according to Steck [23]. Note that the new bands appearing above bands 5 and 6 in A—C and in all subsequent figures are proteolytic fragments generated by the chymotrypsin treatment of the intact cells [20,26].

to those used in Fig. 1, both in the presence and absence of ghosts, TPCK completely inhibited chymotrypsin activity (data not shown). Furthermore, identical results to those shown in Fig. 1 were obtained from ghosts derived from cells incubated with chymotrypsin which had been pretreated with TLCK, a specific trypsin inhibitor that would have inactivated any trypsin impurity possibly present in our chymotrypsin reagent. The possibility that contaminating plasma proteases might account for our results was ruled out by incubating intact red cells with 1 mM PMSF for 5 min at 37°C, washing the cells three times in 15—20 vols. of 310 mosM sodium phosphate (pH 7.4) without PMSF and lysing the cells in lysis buffer without PMSF. Results identical to those of Fig. 1A were obtained. If there were any plasma proteases present in our system, they would have been inactivated prior to lysis when the cells were intact and could not cause proteolysis during and after hemolysis. From these results and the fact that identical results were obtained when leukocytes and platelets had been removed from the blood plasma prior to our experiments (see Experimental procedures), it is clear that band 2.1 proteolysis

and the formation of 3′ is not caused by a residue of exogenous chymotrypsin or trypsin, or by white cell or plasma proteases, but by some other cellular protease(s).

Time course and pH profile of proteolysis

When ghosts prepared from chymotrypsin-treated red cells are incubated at 4° C for extended periods of time in lysis buffers at pH values between 5.5 and 8.5, the rate of proteolysis increases with increasing pH. At pH 5.5 no proteolysis occurs even after 96 h (data not shown), at pH 6.5 (Fig. 2A) the amount of 3′ steadily increases from 0 to 96 h, and at pH 7.6 (Fig. 2B) and pH 8.5 (Fig. 2C) the rates of formation of 3′ plateau after 72 and 48 h, respectively. Inspection of the band 2.1 to 2.6 region over the 96-h time period reveals a series of consecutive proteolytic events which is most apparent in the pH 8.5 preparation. Band 2.1 begins to disappear after 5 h, followed by band 2.2 after 10 h, and bands 2.3 and 2.6 after 24 h. Bands 2.4 and 2.5 appear to increase from 0 to 5 h, plateau, and then decrease at 48 h. In addition, band 4.1, the 60 000 dalton chymotryptic fragment of band 3 [20], band 5 and band 7

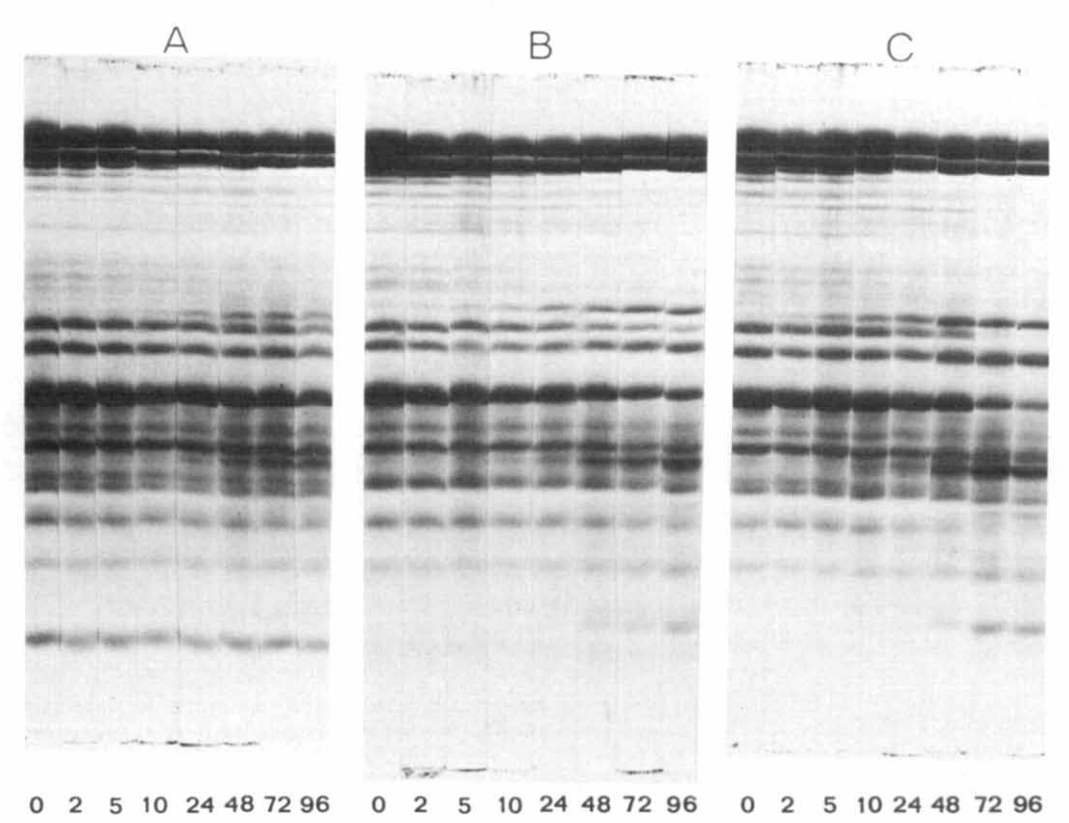
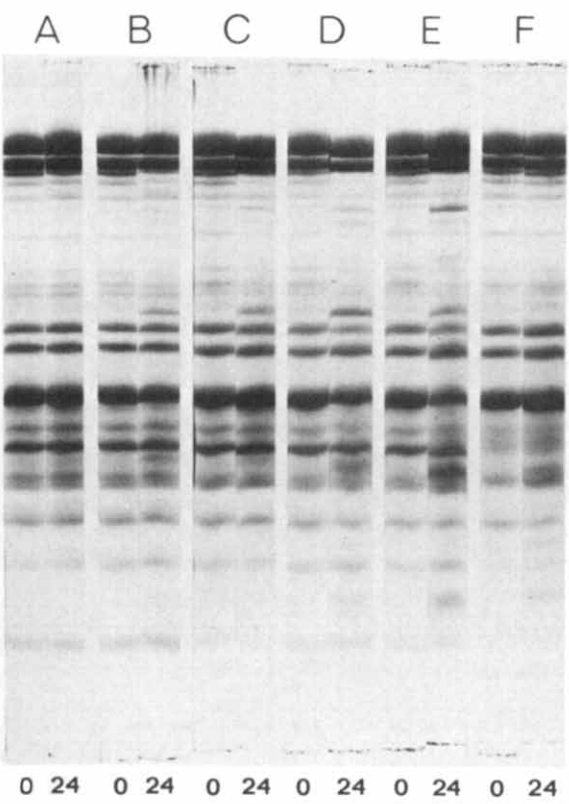


Fig. 2. Time course of proteolysis. Erythrocytes pretreated with α-chymotrypsin were lysed and washed in 20 mosM sodium phosphate (pH 7.6) and then washed three additional times in 20 mosM sodium phosphate at pH 6.5 (A), pH 7.6 (B) or pH 8.5 (C). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. See Fig. 1 for labelling of bands.

decrease in intensity, while proteins of 41 000, 38 000, 27 000 and 18 000 daltons increase in intensity.

From these pH time courses, 24 h was chosen as a suitable incubation period for extending our pH profile to higher pH values (Fig. 3). Above pH 8.5 the rate of proteolysis decreases though at pH 9.5 there is a sharp increase in band 2.4 and in two of the lower molecular weight fragments (41 000 and 18 000) over a 24 h period (Fig. 3E). This may indicate the presence of a series of proteases with different pH maxima or may indicate that at pH 9.5 the polypeptide which generates band 2.4 is more accessible for proteolytic digestion.

Proteolysis is not observed in spectrin-depleted inside-out vesicles which have been washed twice in pH 7.6 lysis buffer and incubated at 4° C (Fig. 4). The



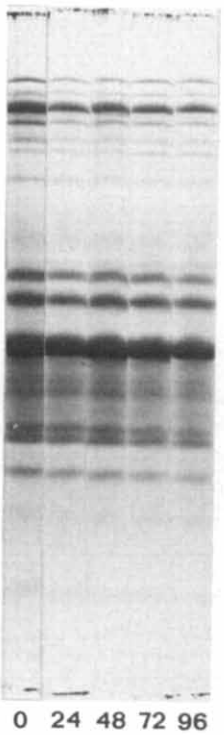


Fig. 3. pH profile of proteolysis. Erythrocytes pretreated with α-chymotrypsin were lysed and washed in 20 mosM sodium phosphate (pH 7.6) and then washed three additional times in 20 mosM sodium phosphate at pH 5.5 (A), pH 6.5 (B), pH 7.6 (C), pH 8.5 (D), pH 9.5 (E) or pH 10.5 (F). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. See Fig. 1 for labelling of bands.

Fig. 4. Proteolysis in spectrin-depleted inside-out vesicles. Ghosts were prepared from chymotrypsin-treated erythrocytes in 20 mosM sodium phosphate (pH 7.6). Spectrin-depleted inside-out vesicles were prepared from these ghosts as previously described [4], washed twice in 20 mosM sodium phosphate (pH 7.6) and incubated at 4°C. Aliquots were removed for gel electrophoresis at the time periods (h) indicated. See Fig. 1 for labelling of bands.

protease molecules may be loosely attached to the inside of the ghost membrane from where they might be removed by continuous washes in lysis buffer after the membranes are inverted.

Effects of divalent cations

Cells lysed in the presence of 1 mM $CaCl_2$ showed a marked increase in the amount of band 2.3 and complete absence of band 2.1 at zero time; after 24 h at 4°C, band 2.3 decreases in intensity and 3′ forms just as in the absence of Ca^{2+} (Fig. 5A and B). Lysis in the presence of either 1 mM EDTA (Fig. 5C) or 1 mM $ZnCl_2$ (Fig. 5D) resulted in partial inhibition of proteolysis and a loss of band 8 in the Zn^{2+} preparation. Partial inhibition of proteolysis also occurred when the cells were lysed in either 1 mM $MgCl_2$ or $MnCl_2$ (Fig. 5E and F) and a protein aggregate formed when the lysis buffer contained 1 mM $FeCl_2$ (Fig. 5G). It is not clear whether proteolysis was inhibited by Fe^{2+} or if 3′ formed and was subsequently cross-linked. Throughout these experiments, it was observed that cells lysed and washed in the presence of 1 mM concentrations of the divalent cations were more difficult to free of hemoglobin than control ghosts.

To test whether the Ca^{2+}-induced effects require the presence of the divalent cation during the initial lysis step as suggested by Carraway et al. [11], all

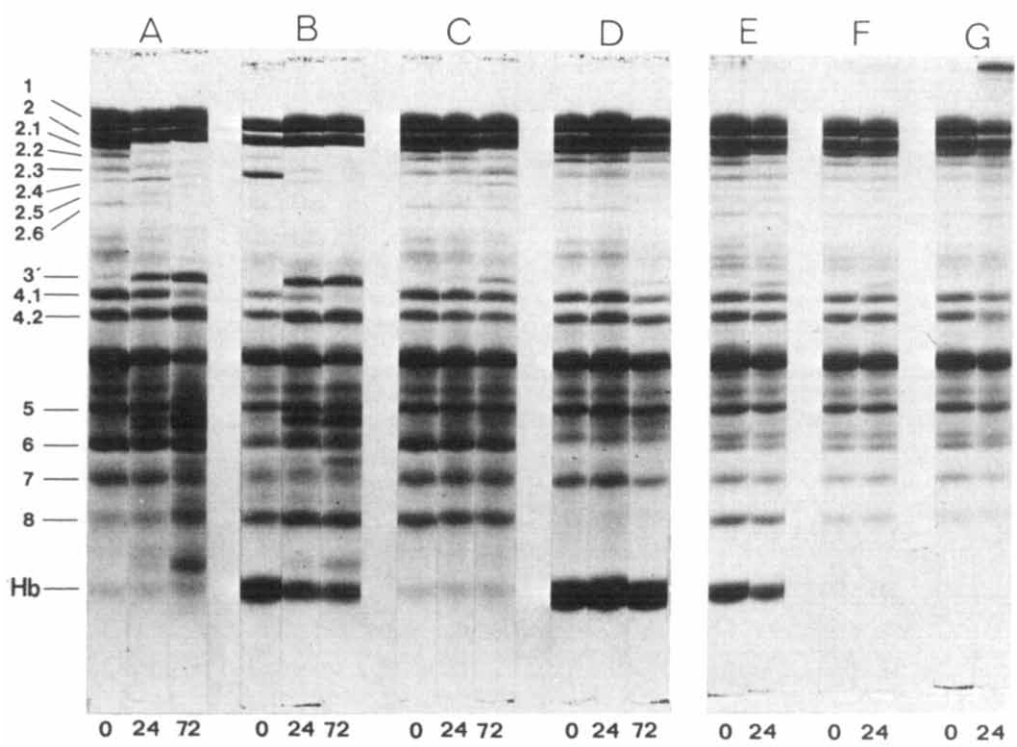


Fig. 5. Effect of divalent cations. Erythrocytes pretreated with α-chymotrypsin were lysed and washed in 20 mosM sodium phosphate (pH 7.6) in the absence (A) or presence of 1 mM $CaCl_2$ (B), 1 mM EDTA (C), 1 mM $ZnCl_2$ (D), 1 mM $MgCl_2$ (E), 1 mM $MnCl_2$ (F) or 1 mM $FeCl_2$ (G). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. Hb, hemoglobin; band labelling according to Steck [23].

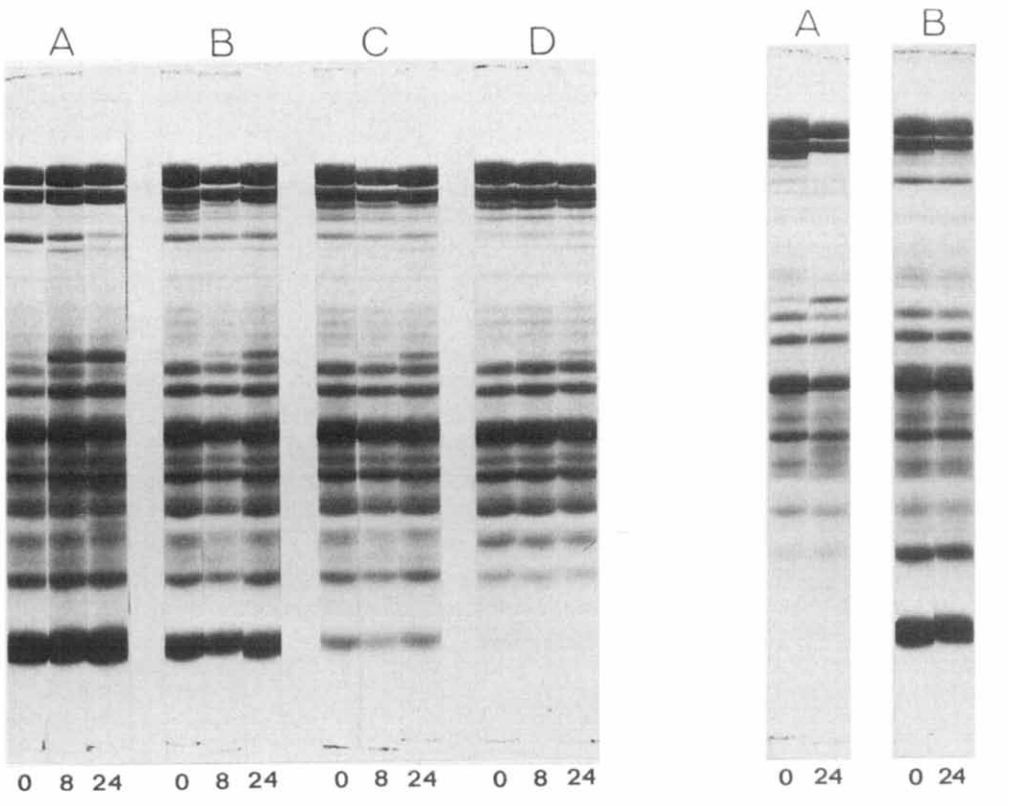


Fig. 6. Effect of adding Ca^{2+} in successive washes. Erythrocytes pretreated with α-chymotrypsin were lysed in 20 mosM sodium phosphate (pH 7.6) containing 1 mM $CaCl_2$ in all washes (A), in the second, third and fourth washes (B), in the third and fourth washes (C), or in no washes (D). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. See Fig. 5 for labelling of bands.

Fig. 7. Proteolysis in the presence of both Ca^{2+} and PMSF. Erythrocytes pretreated with α-chymotrypsin were lysed and washed in 20 mosM sodium phosphate (pH 7.6) in the absence (A) or presence of 1 mM $CaCl_2$ and 1 mM PMSF (B). Ghost preparations were incubated at 4°C and aliquots were removed for gel electrophoresis at the time periods (h) indicated. See Fig. 5 for labelling of bands.

samples were lysed in buffer containing 1 mM $CaCl_2$ in all washes (Fig. 6A), in the second, third and fourth washes (Fig. 6B), in the third and fourth washes (Fig. 6C), or in no washes (Fig. 6D). It is clear that the Ca^{2+}-induced effects are most pronounced when Ca^{2+} is present from the moment of initial hemolysis. Similar experiments with the other divalent cations also indicated that the induced effects, whether stimulatory or inhibitory, were most apparent if the effector was present in the first lysis wash (data not shown).

If cells were lysed in buffer containing 1 mM of both $CaCl_2$ and PMSF, the Ca^{2+}-induced disappearance of band 2.1 and formation of band 2.3 at zero time still occurred, but there was no further proteolysis of band 2.3 or any other polypeptide in that region, and 3′ did not form with time at 4° C (Fig. 7). This indicates the presence of at least two types of red cell proteases. The enzyme which converts 2.1 to 2.3 is Ca^{2+}-stimulated (Figs. 5 and 6) and insensitive to

PMSF (Fig. 7B) whilst the enzyme(s) responsible for the conversion of 2.3 to 3′ (Fig. 7B) or the cascade from 2.1 to 3′ (Fig. 1C) are inhibited by PMSF.

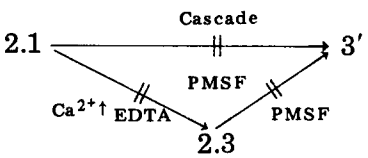
Pretreatment of erythrocytes with proteolytic inhibitors

In view of the fact that the entire band 2.1 to 2.6 family is present in ghosts when 1 mM PMSF and 1 mM EDTA are included in the lysis buffer, we asked what effect pretreatment of erythrocytes with these inhibitors would have upon bands 2.2 to 2.6.

Erythrocytes pretreated with 1 mM PMSF and 1 mM EDTA for 20 h at 4°C were lysed and washed in lysis buffer containing 1 mM PMSF and 1 mM EDTA. Ghost preparations were denatured in SDS preheated to 37°C or 70°C [21] and electrophoresed as described in Experimental procedures. SDS-polyacrylamide gels of these ghost preparations were identical to those of ghosts obtained from non-pretreated erythrocytes. Preheating of the SDS to 70°C had no effect upon the SDS-polyacrylamide gel electrophoretic protein pattern. Bands 2.2 to 2.6 are always present independent of whether precautions are taken to stop proteolysis during ghost preparation and solubilization.

Discussion

We have demonstrated that endogenous proteolysis activity is responsible for the in vitro cleavage of the erythrocyte spectrin binding proteins and the formation of 3′, a membrane-bound fragment that contains an active spectrin binding site. Our results indicate that this can occur through two enzymatic pathways: a cascade of consecutive proteolytic cleavages inhibited by PMSF or a Ca^{2+}-stimulated, PMSF-insensitive, EDTA-inhibited cleavage of band 2.1 to band 2.3, followed by digestion to band 3′ by PMSF-inhibited enzymes (possibly of the first pathway).



This model is supported by our findings that PMSF is capable of inhibiting all proteolysis in the absence of exogenous Ca^{2+} (Fig. 1C) and that the addition of Ca^{2+} along with PMSF blocks proteolysis beyond band 2.3 (Fig. 7). These effects and the other concomitant changes in membrane protein composition are summarized schematically in Fig. 8.

Our findings are consistent with previous reports that proteolytic activity is stimulated by Ca^{2+} [10,11,13,14] and inhibited to varying degrees by PSMF, EDTA and Zn^{2+} [9,11,14]. It is likely that the Ca^{2+}-induced formation of a 174 000 dalton polypeptide and loss of a 91 000 dalton polypeptide reported by Tökes and Chambers [9] are the same alterations in bands 2.3 and 4.1, respectively, which we report here, and that the proteolytic fragment migrating in the band 3 region observed by Quirk et al. [13] is band 3′. Since band 2.1 is degraded and a large amount of 3′ is initially present in proteolipid shells formed by Triton X-114 extraction of intact erythrocytes [5,6], proteolytic

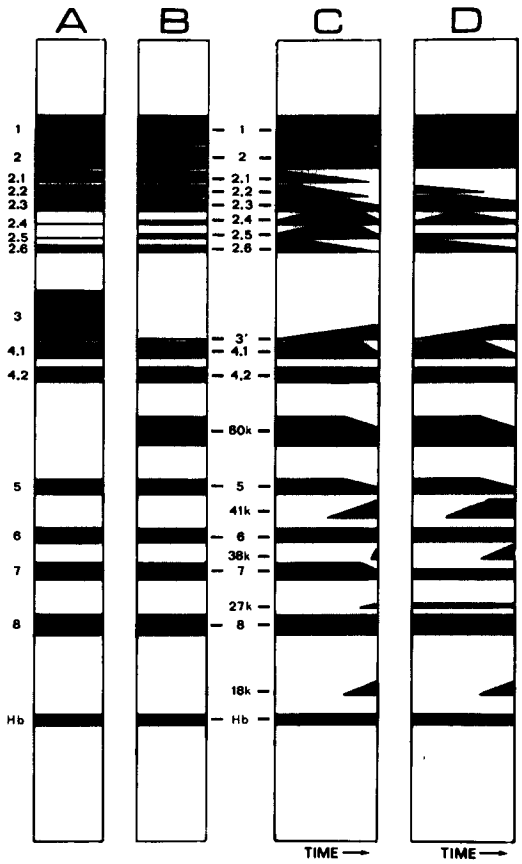


Fig. 8. Summary of proteolytic events. A and B are drawings of gel electrophoretic patterns obtained from erythrocyte ghosts prepared from untreated and chymotrypsin-treated erythrocytes, respectively; C and D are schematic representations of the time-dependent alterations in the membrane proteins illustrated in B when cells are lysed and washed in the absence (C) or presence (D) of 1 mM $CaCl_2$ and are incubated at 4°C. In C and D, the horizontal direction represents a logarithmic time scale ranging from 0 to 4 days. The height of each band is proportional to the amount of that component except for bands 2.2 and 2.3 which were thickened for clarity.

activity appears to be stimulated by Triton. The increased rate of proteolysis induced by the non-ionic detergent NP-40 [9] may occur through the same mechanism. Others, unaware of the 2.1 family of proteins, have attributed the Ca^{2+}-induced formation of band 2.3 to adsorption of cytosol proteins [12,14]: our data support the view that it is a proteolytic product of band 2.1. Furthermore, the fact that cleavage of band 2.1 and the formation of band 2.3 is observed in fresh ghosts derived from intact red cells which were incubated with Ca^{2+} and ionophore A23187, washed extensively without Ca^{2+}, and lysed without Ca^{2+} [13,14,22] supports our view that the proteases must originate from within the erythrocyte.

The fact that all of the spectrin binding proteins are present in ghosts even when protease inhibitors are included before and during cell lysis implies that all of them are also present in the native erythrocyte. Their in vivo presence may be the result of post-translational cleavage of a parent polypeptide by

proteolytic enzymes similar to those we have observed in vitro. Why the red cell requires several different spectrin binding proteins remains to be ascertained. Since the polypeptides in the band 2.1 to 2.6 region have been shown to change with age [25], their interconversion during the 120-day life span of the mature human erythrocyte may play a role in red cell senescence and in regulating the rigidity and shape of the erythrocyte membrane.

Acknowledgements

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