Calpain-Calpastatin and fusion

Fusibility of erythrocytes is determined by a protease-protease inhibitor [calpain-calpastatin] balance

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Rat erythrocytes fuse when treated with the membrane mobility agent, 2-(2-methoxyethoxy)ethyl-*cis*-8-(2-octylcyclopropyl)octanoate (A_2C) and Ca^{2+}, whereas human cells do not. Membrane proteolysis promoted by calpain is required for rat cell fusion [(1986) Eur. J. Biochem., in press]. Human calpain induced a selective proteolysis in both the human and rat erythrocyte ghosts (mainly band 4.1 in the human, band 4.1 and band 3 in the rat cell) and rendered them fusible. Calpastatin (calpain inhibitor) prevented A_2C-induced fusion in both ghosts, via inhibition of proteolysis. The human erythrocyte has excess calpastatin and resists A_2C-promoted fusion. A regulatory role of calpastatin in membrane fusion is thus indicated.

Membrane fusion *Membrane proteolysis* *Calpain/calpastatin* *Erythrocyte membrane* *Membrane mobility agent* *Fusogenic agent*

1. INTRODUCTION

Membrane fusion is important for diverse cellular processes such as fertilization, myoblast fusion and virus entry into cells [1–3]. It is induced experimentally by certain viruses and chemical agents [1–6]. The membrane mobility agent, 2-(2-methoxyethoxy)ethyl-*cis*-8-(2-octylcyclopropyl)-octanoate (A_2C) is an inducer of membrane fusion and has been useful in defining stages and factors involved in this process [6,7].

We have previously found differences among erythrocytes of various species in the response to A_2C [8]. Rat erythrocytes fuse readily when treated with A_2C and Ca^{2+}, but human cells do not [8,9]. We have shown fusibility to be correlated with Ca^{2+}-induced membrane protein degradation, which occurs in the rat erythrocyte but not in the human cell [9]. Using rat erythrocyte ghosts, we have demonstrated that the membrane protein degradation required for rat erythrocyte fusion is due to the Ca^{2+}-activated, cytoplasmic thiol-protease, calpain I [9,10].

Calpains are widely distributed in avian and mammalian tissues, including erythrocytes [11]. A calpain inhibitor, calpastatin, has also been found in a variety of cells in widely varying amounts [11,12]. Calpastatin activity has been identified through the inhibition of calpain-induced hydrolysis of several soluble proteins, such as casein and globin [11–13]. Thus defined, calpain activity in rat erythrocytes is greater than calpastatin activity, whereas in human erythrocytes calpastatin activity is greater than that of calpain [11].

In view of the differences noted between rat and human erythrocyte fusibility [8–10], we have investigated the effect of calpastatin on A_2C-induced membrane fusion. We show that calpastatin prevents fusion of human and of rat erythrocyte membranes through inhibition of calpain-dependent membrane proteolysis. An activity of

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calpastatin on a cellular level is thus demonstrated and a regulatory role in membrane fusion events is indicated.

2. MATERIALS AND METHODS

2.1. *Erythrocyte ghosts*

Heparinized blood from healthy humans and rats (Charles River derived rats) was centrifuged, the plasma and buffy coat removed, and the erythrocytes washed with 150 mM NaCl. Hemoglobin-free ghosts were prepared by hemolyzing washed erythrocytes in 5 mM phosphate buffer (pH 8.2), followed by washing with 10 mM NaCl.

2.2. *Calpain and calpastatin*

Human erythrocytes were hemolyzed in 12 vols of 5 mM Tris-HCl (pH 7.4), 1.0 mM EGTA and 0.5 mM dithiothreitol (DTT) (buffer A). Membrane-free hemolysate (obtained by centrifugation of the lyzed cells at 40000 × *g* for 30 min) was used for the isolation of calpain and calpastatin according to [11,12] with slight modifications. A DEAE-cellulose (Sigma, DE-52) column was prewashed with 50 mM NaCl/buffer A. After loading of the column with the hemolysate containing 50 mM NaCl, the column was washed with 50 mM NaCl/buffer A until free of hemoglobin. A fraction known to contain calpain and calpastatin was then eluted with 150 mM NaCl/buffer A [12]. This fraction was dialyzed against 0.1 mM EGTA, lyophilized, resuspended in a small volume of 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA and 0.5 mM DTT (buffer B) and centrifuged at 40000 × *g* to clarify the solution. The concentrated fraction was loaded on an Ultrogel AcA 34 (LKB, Sweden) and fractions eluted with buffer B. Aliquots were analyzed for calpain activity, using casein as substrate [11]. The fractions containing calpain activity were pooled, lyophilized and resuspended in buffer B. For further purification of calpain, the active fraction was chromatographed on a Blue Sepharose CL-6B column, prewashed with 50 mM NaCl/buffer B. Fractions were eluted with 1.0 M urea/buffer B. Fractions containing calpain activity were pooled and dialyzed with buffer B and loaded on DEAE Bio-Gel A, prewashed with buffer B. Fractions were eluted with a linear gradient of 0–250 mM NaCl. The pooled active fractions were lyophilized.

For the determination of calpastatin activity, aliquots from the Ultrogel column fractions were heated at 100°C for 15 min to destroy calpain activity and centrifuged. Supernatant was added to a calpain-containing fraction and the decrease in calpain activity determined [11,12]. Fractions containing calpastatin were pooled and further purified by rechromatography on a DE-52 column. After sample loading, the DE-52 column was washed with 80 mM NaCl/buffer B, followed by 100 mM NaCl/buffer B for the elution of calpastatin. The fractions containing calpastatin were pooled, dialyzed, lyophilized and resuspended in buffer B. This fraction was heated as above, centrifuged and supernatant used as calpastatin solution. Aliquots of calpain and calpastatin were analyzed by SDS-acrylamide gel electrophoresis [11].

2.3. *Membrane proteolysis and fusion*

Ghosts were suspended in buffer containing 135 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.5 mM DTT, 1.0 mM $CaCl_2$ and 40 mg/ml dextran (T-70, Pharmacia) (buffer C). Ghost suspensions were incubated with a solution of calpain or calpain and calpastatin, centrifuged, resuspended in buffer C and incubated with $\dot{A}_2C$ [9]. Aliquots were examined microscopically for fusion [9]. Ghosts were then mixed with 5 mM EDTA, centrifuged, solubilized and analyzed by SDS-acrylamide gel electrophoresis [9].

3. RESULTS

Calpain and calpastatin, derived from human erythrocytes, were analyzed in fractions eluted from the Ultrogel AcA 34 column. Calpain activity was eluted at a position of about 110–120 kDa, and calpastatin at about 240–280 kDa (fig.1A). Calpain was further purified on Blue Sepharose CL-6B (not shown) followed by DEAE Bio-Gel A (fig.1B). Calpastatin was further purified on a DE-52 column (not shown). Electrophoresis of the calpain-containing fraction on SDS-acrylamide showed bands of 80 and 30 kDa and that of calpastatin showed a band of 70 kDa (fig.1C). The

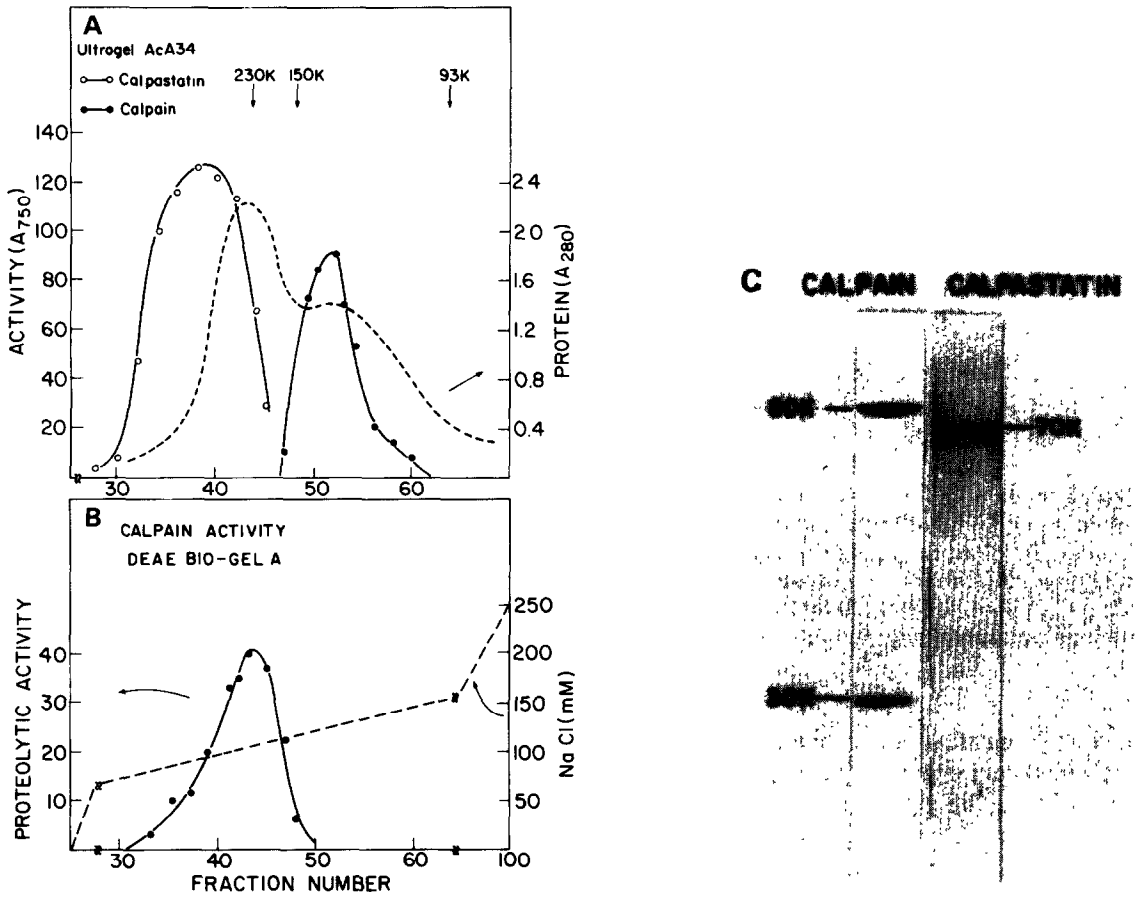

Fig.1. (A) Elution profile of calpain and calpastatin on a column of Ultrogel AcA 34. Hemolysate from 120 ml of erythrocytes was first eluted from a DE-52 column, then loaded on the Ultrogel column (see section 2). Fractions of 1.4 ml were collected. Protein (A_{280}) refers to the absorbance of eluted fractions at 280 nm, for the estimation of protein concentration. Activity (A_{750}) refers to a change in absorbance at 750 nm. For calpain, one unit of activity corresponds to an increase of 1.0 A unit at 750 nm after 30 min incubation of casein and calpain, as defined by Hatanaka et al. [11]. For calpastatin, one unit of activity corresponds to a decrease of 1.0 A unit (i.e. a decrease of one unit of calpain activity) at 750 nm when calpastatin is added to the calpain-containing fraction [11,12]. (B) Elution profile of calpain chromatographed on DEAE Bio-Gel A (fractions and units as in A). (C) SDS-polyacrylamide gel electrophoretic patterns of purified calpain and calpastatin.

chromatographic behavior of the active fractions and the electrophoretic analysis are in agreement with the properties reported for calpain I and calpastatin [11,12].

Human erythrocyte ghosts were incubated with DTT, Ca^{2+} and calpain, with or without calpastatin, followed by A_2C, as described in section 2. Ghosts incubated without calpain did not fuse when treated with A_2C (fig.2A). Calpain-treated ghosts fused upon the addition of A_2C to yield multicellular bodies (fig.2B). In contrast, calpain- and calpastatin-treated ghosts did not fuse upon the addition of A_2C (fig.2C). Similar inhibition of fusion was obtained by leupeptin, whereas fusion did occur in the presence of phenylmethylsulphonyl fluoride (not shown). Rat erythrocyte ghosts behaved in a similar way. Ghosts pretreated with calpain fused upon the addition of A_2C, whereas ghosts pretreated with calpain and calpastatin did not fuse (not shown).

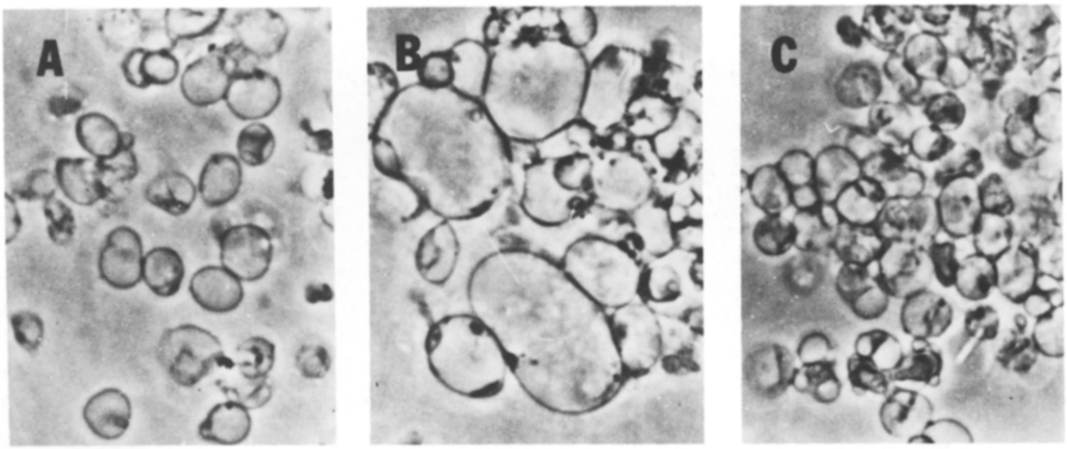

Fig.2. Micrographs showing fusing and non-fusing human erythrocyte ghosts. Calpain or calpain and calpastatin were first incubated at 30°C for 20 min, then added to ghost suspensions in buffer C (8 units calpain and 15 units calpastatin/ml of 4% ghost suspension, containing 0.2 mg membrane protein). Ghost suspensions were then incubated at 37°C for 40 min, centrifuged, resuspended in buffer C and mixed with half a volume of A_2C suspension (prepared by sonication of 0.5 μl of A_2C/ml 150 mM NaCl for about 30 s [6]). A_2C-ghost suspensions were reincubated at 37°C. Aliquots were photographed after 60 min of incubation. Ghosts were incubated without calpain (A), with calpain (B), with calpain and calpastatin (C).

Electrophoretic profiles of proteins, isolated from human erythrocyte ghosts treated with A_2C, are shown in fig.3. The profile of proteins from control, non-fusing ghosts (incubated in buffer without calpain, followed by A_2C) is shown in fig.3A. In fusing ghosts (calpain-treated, followed by A_2C) band 4.1 disappeared, with slight changes in spectrin bands 1, 2 and in band 3, accompanied by the appearance of bands of lower molecular masses (fig.3B). In non-fusing ghosts (calpain- and calpastatin-treated, or calpain- and leupeptin-treated, followed by A_2C) the profiles were similar to that of the control shown in fig.3A, i.e. 4.1 remained and no changes were visible in bands 1, 2 and 3 (fig.3C,D).

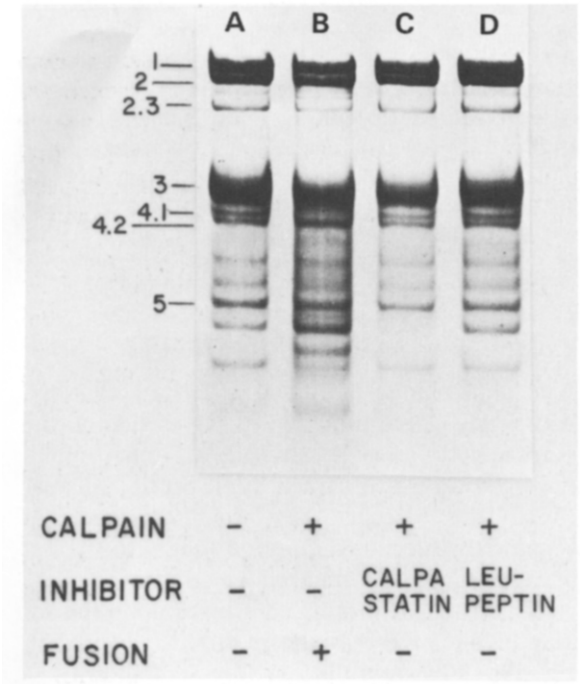

Fig.3. SDS-polyacrylamide gel electrophoretic patterns of membrane proteins isolated from fusing and non-fusing human erythrocyte ghosts. Ghost suspensions (4% in buffer C) were incubated at 37°C for 40 min, centrifuged and further treated with A_2C, as described in fig.2. Ghosts incubated without calpain (A), with 8 units calpain/ml ghost suspension (B), with 8 units calpain and 15 units calpastatin (C), with 8 units calpain and 1.0 mM leupeptin (D).

Ghosts incubated with DTT, Ca^{2+} and calpain, but without A_2C, underwent proteolysis similar to that shown in fig.3B, but did not fuse (not shown). A more extensive proteolysis was achieved by longer incubation of the human erythrocyte ghosts

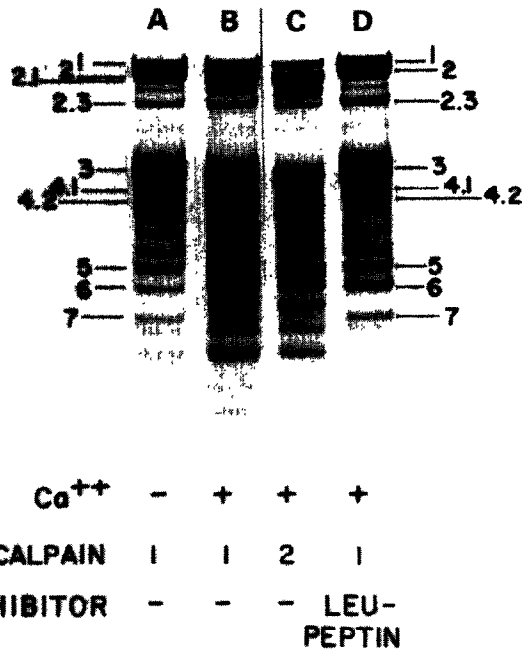

Fig.4. SDS-polyacrylamide gel electrophoretic patterns of membrane proteins isolated from calpain-treated human erythrocyte ghosts. Ghost suspensions (4% in buffer C) were incubated at 37°C for 120 min, with 8 units calpain and $MgCl_2$ (instead of $CaCl_2$)/ml ghost suspension (A), 8 units calpain (B), 16 units calpain (C), 8 units calpain and 1.0 mM leupeptin (D). Calpain 1 refers to 8 units calpain/ml ghost suspension and calpain 2 to 16 units calpain/ml ghost suspension.

with the same or higher amounts of calpain (fig.4B,C). Under these conditions, the changes in bands 1, 2 and 3 and the appearance of bands of lower molecular masses were more noticeable than those shown in fig.3B, in addition to the disappearance of band 4.1. When Mg^{2+} was substituted for Ca^{2+} or when calpain activity was inhibited, no changes were observed in bands 1, 2, 3 and 4.1 (fig.4A,D). The ghosts undergoing extensive proteolysis did not fuse in the absence of A_2C and usually disintegrated to small vesicles upon treatment with A_2C.

4. DISCUSSION

We have previously found that rat erythrocytes fuse when treated with A_2C and Ca^{2+}, whereas human cells do not. Rat erythrocyte ghosts, pretreated with calpain and Ca^{2+}, undergo fusion upon the addition of A_2C [8–10]. Here, we show that human erythrocyte ghosts can also be induced to fuse by A_2C, provided they are pretreated with Ca^{2+} and calpain, and are free of calpastatin.

Calpains have been shown to degrade certain cellular, membrane and cytoskeletal proteins [11–19]. Neither the nature of calpastatin activity towards cellular constituents nor the cellular roles of calpastatin are known. Calpastatin is shown here to inhibit calpain-induced membrane proteolysis and prevent A_2C-induced membrane fusion in both human and rat erythrocyte ghosts. Calpastatin may thus have a regulatory role in the function of membranes via modulation of calpain-induced proteolysis of membranes and/or cytoskeletal proteins.

Membrane proteolysis is necessary, but not sufficient by itself to induce fusion. The present results point to a selective and limited proteolysis, i.e. mainly degradation of band 4.1, as prerequisite for A_2C-induced fusion [though partial proteolysis of band 2.1 may also be involved (preliminary observations)]. Such proteolysis may free restrained membrane components and render them potentially mobile. The actual mobility must be enhanced with a reagent such as A_2C, creating protein particle-poor, fusion-potent areas [8,9].

Human calpain used here induced protein degradation in both human and rat erythrocyte membranes and rendered them fusible. Alterations in membrane proteins, however, were not identical in the human and rat erythrocyte. Calpain induced degradation of band 4.1 in both the human and the rat erythrocyte. In the rat, in addition to band 4.1 degradation, calpain also induced limited proteolysis of band 3, the degradation of both being associated with fusibility of rat erythrocyte ghosts (unpublished). This may be due to differences in membrane structure and/or to differences in target proteins and in substrate specificity. The fact that both membranes were rendered fusible indicates that the same consequence may be achieved in membranes having dissimilar structure and substrate specificity. The protein(s) and the amino acid sequences subject to attack by the human and rat enzymes are currently under study.

In conclusion, the human erythrocyte membrane is capable of undergoing fusion. The lack of

fusibility is not because of resistant membrane structure, but is due to the activity of the protease inhibitor, calpastatin. Cells are known to vary in fusibility [20,21]. Differences in the balance of protease and protease inhibitor activity may account for some cases of poor fusibility.

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