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Evidence for Membrane-Associated Calpain I in Human Erythrocytes. Detection by an Immunoelectrophoretic Blotting Method Using Monospecific Antibody†

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ABSTRACT: Low and high Ca^{2+}-requiring forms of Ca^{2+}-dependent cysteine proteinase are known as calpain I and calpain II, respectively. We have obtained, for the first time, monospecific antibodies for calpain I and for calpain II. Using these antibodies and an electrophoretic blotting method, we have found that a small, but reproducible, amount of calpain I was associated with human erythrocyte membranes while the bulk of the protease was contained in the cytosol. Most of membrane-associated calpain I was extractable with 1% Triton X-100, but not with 0.1% detergent. In the presence of 0.1 mM Ca^{2+} and 5 mM cysteine, membrane-associated calpain I degraded the membrane protein band 4.1 preferentially and band 3 protein only slowly. The Ca^{2+}-induced autodigestion of the membrane preparation was inhibited by leupeptin but not by a cytosolic calpain inhibitor, calpastatin, added to the incubation medium. No calpain II was detected in either erythrocyte cytosol or membranes when anti-calpain II antibody was used under the same conditions as those for the detection of calpain I.

The Ca^{2+}-dependent cysteine proteinase, collectively called calpain[1] [EC 3.4.22.17], has been found mainly in the cytosolic fraction of various mammalian and avian cells (Guroff, 1964; Huston & Krebs, 1968; Dayton et al., 1976; Phillips & Jakâbovâ, 1977; Murachi et al., 1981b; Murakami et al., 1981; Nelson & Traub, 1982). Although physiological functions of such widely distributed calpains are still obscure, recent works have shown that this enzyme degrades native cytoskeletal proteins including high molecular weight microtubule-associated proteins (Sandoval & Weber, 1978), actin-binding proteins (Davies et al., 1978; Truglia & Stracher, 1981), and

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[1] Recommended name is given by the Nomenclature Committee of the International Union of Biochemistry (1981). Calpain I denotes a low Ca^{2+}-requiring form and calpain II a high Ca^{2+}-requiring form. The 80K and 30K proteins represent subunits of calpain having molecular masses of 70 000–85 000 and 25 000–30 000 daltons, respectively.

0006-2960/84/0423-3272$01.50/0

various intermediate filaments (Pant et al., 1979; Zimmerman & Schlaepfer, 1982; Nelson & Traub, 1982). Proteolysis of high molecular weight microtubule-associated proteins and actin-binding proteins causes an irreversible loss of polymerization of tubulin and actin, respectively (Sandoval & Weber, 1978; Davies et al., 1978). Therefore, it is speculated that calpain would have an important role in regulating the assembly of cytoskeletal proteins. In erythrocytes, too, the cytoplasmic surface membrane is lined with a deformable meshwork that constitutes a cytoskeleton. Band 4.1,[2] together with spectrin and actin, is the major constituent of this cytoskeleton (Branton et al., 1981). Ca^{2+}-dependent loss of membrane proteins including band 4.1 has frequently been observed in intact erythrocytes and ghost preparations (Triplett et al., 1972; King & Morrison, 1977; Anderson et al., 1977; Allen & Cadman, 1979; Lorand et al., 1983). An earlier report from this laboratory has shown that isolated erythrocyte cytosol calpain degrades preferentially band 3 and band 4.1 proteins (Murakami et al., 1981). A similar change also obtained by autolysis of the isolated membranes suggests a possibility of the association of calpain with membranes.

Two forms of calpain have been found that differ in Ca^{2+} requirement, calpain I requiring low Ca^{2+} and calpain II requiring high Ca^{2+} (Dayton et al., 1981; Mellgren, 1980; Murachi et al., 1981b). These calpains, separated by anion-exchange chromatography, exist in a variable proportion in various tissues and cells (Murachi et al., 1981a). However, the cytosol of erythrocytes contained only calpain I, whereas calpain II was not identified by the chromatographic procedures (Murakami et al., 1981; Hatanaka et al., 1983). It was therefore questioned whether or not calpain I is also present in the membrane fractions of human erythrocytes and whether or not the membranes contain calpain II.

In the present study evidence has been obtained by an immunoelectrophoretic blotting method for the association of a small but distinct portion of calpain I with human erythrocyte membranes. The same method has also confirmed that calpain II is identified neither in erythrocyte membranes nor in the cytosol. A possible physiological significance of membrane-associated calpain I is discussed.

Experimental Procedures

Materials. PMSF[3] was obtained from Sigma, CNBr-activated Sepharose 4B from Pharmacia, Bio-Beads SM-2 from Bio-Rad, nitrocellulose paper (membrane filter BA 85) from Schleicher & Schuell, and peroxidase-conjugated goat anti-rabbit IgG from Cappel Laboratories, Inc. Leupeptin was a generous gift from Dr. H. Umezawa, Research Institute for Microbial Chemistry, Tokyo, Japan.

Erythrocyte Cytosol and Membranes. Leukocyte- and platelet-free human erythrocytes were obtained as previously reported (Murakami et al., 1981) and hemolyzed with 10 volumes of 20 mM Tris-HCl buffer containing 1 mM EGTA, 1 mM EDTA, and 5 mM 2-mercaptoethanol, pH 7.5 (buffer A). The hemolyzate was centrifuged at 15000*g* for 30 min, and both cytosol and membranes were saved. The hemolyzate supernatant was dialyzed against buffer A overnight and used as "erythrocyte cytosol". Hemoglobin-free membranes were prepared by the method of Dodge et al. (1963), except that buffer A was used. The membranes were finally washed with buffer A, containing 50 mM NaCl, of the same volume as that of the membranes. Besides the "membranes" thus obtained, the "final washing" was also saved for the examination of possible contamination with calpain from the cytosol fraction. "Triton membrane skeletons" were prepared by the method of Yu et al. (1973). Briefly, the membranes were extracted with 1% Triton X-100 for 10 min at room temperature, and the membrane skeletons were pelleted at 100000*g* for 30 min. The supernatant was saved as "Triton extracts". After one washing, the membrane skeletons were suspended in buffer A. In order to guard against possible proteolytic degradation, 1 mM PMSF was added to each preparation; PMSF was known to have no effect on calpain activity.

Electrophoresis. Electrophoresis was done according to the procedure of Laemmli (1970) with 7.5% or 12.0% resolving gel and 3.0% stacking gel. Gels were stained with Coomassie brilliant blue R-250. For preparative runs, chromatographically purified calpains were loaded, and after the electrophoresis, the gels were soaked in cold 0.5 M NaCl for 10 min and further kept in crushed ice for 1 h to make bands for 80K and 30K subunits visible. The proteins thus localized were extracted from the gels with 0.05 M NH_4HCO_3 containing 0.05% SDS overnight at room temperature and dialyzed against distilled water (Weber & Osborn, 1975). They were found to be homogeneous on SDS gel electrophoresis and used as ligands in the affinity column for IgG purification.

Immunoelectrophoretic Blotting Procedures. Proteins were first subjected to electrophoresis on 12.0% polyacrylamide gels as described above and were transferred from the gels to nitrocellulose paper according to the method of Towbin et al. (1979). Blotted proteins were incubated with IgG monospecific for the calpain I 80K subunit or IgG monospecific for the calpain II 80K subunit. Calpains were localized on the blotted sheets with horseradish peroxidase conjugated goat anti-rabbit IgG by the method of Hawkes et al. (1982).

Preparation of Antisera. Human erythrocyte calpain I and porcine kidney calpain II were purified as reported elsewhere (Hatanaka et al., 1983; Yoshimura et al., 1983; Sasaki et al., 1983). Antisera against them were raised in rabbits as previously reported (Sasaki et al., 1983).

Purification of Antibodies. The IgG fractions of the antisera against calpain I and calpain II were isolated by conventional ammonium sulfate fractionation and DEAE-cellulose chromatography, respectively. They were further purified by using two steps of affinity chromatography. An affinity column with the 80K subunit of calpain I or calpain II as the ligand was prepared by coupling 2 mg of the protein to 1 g of CNBr-activated Sepharose 4B according to the method of Sasaki et al. (1983). Anti-calpain I IgG solution (60–90 mg of protein in 30 mL) was dialyzed against 0.1 M sodium carbonate buffer, pH 8.3, containing 0.5 M NaCl and preabsorbed on a 1.0 × 5.0 cm column of calpain II 80K subunit–Sepharose, with which IgG cross-reactable to calpain II was removed. The unbound proteins were collected and applied to a 1.0 × 5.0 cm column of calpain I 80K subunit–Sepharose. After unbound proteins were washed out with the above buffer, antibody bound to the column was eluted with 0.2 M glycine hydrochloride buffer, pH 2.3. The pH of the eluent was immediately adjusted to neutrality with 0.1 M NaOH. The solution was then dialyzed against phosphate-buffered saline, pH 7.2. The anti-calpain II IgG fraction was purified in the same way but with the reversed sequence in the use of two affinity columns.

[2] The major membrane proteins are enumerated according to their characteristic electrophoretic mobilities and staining pattern according to Fairbanks et al. (1971), as modified by Steck (1972, 1974).

[3] Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IgG, immunoglobulin G; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

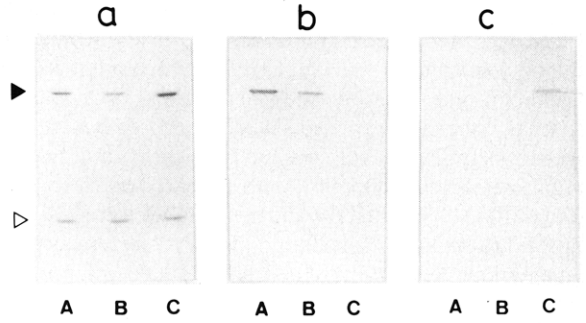

FIGURE 1: Specificity of the affinity-purified anti-calpain I IgG and anti-calpain II IgG. Antigens used were the following: lane A, 3 μg of purified human erythrocyte calpain I; lane B, 3 μg of porcine erythrocyte calpain I; lane C, 3 μg of porcine kidney calpain II. Part a shows the Coomassie blue stained SDS–polyacrylamide gel (12.0%). The proteins on gels were transferred to the nitrocellulose membranes and were depicted by the binding with affinity purified anti-human erythrocyte calpain I IgG (b) or with anti-porcine kidney calpain II IgG (c) using horseradish peroxidase conjugated goat anti-rabbit IgG. Closed arrowhead indicates the position of 80K subunit, and open arrowhead indicates the position of 30K subunit.

Autodigestion of Membranes. Inside out vesicles prepared by the method of Steck & Kant (1974) were autodigested in the presence of 0.1 mM $CaCl_2$, 5 mM cysteine, and 50 mM imidazole hydrochloride buffer, pH 7.2. The digests were analyzed on 7.5% polyacrylamide gel electrophoresis. Calpastatin used was purified from human erythrocytes as previously reported (Takano & Murachi, 1982).

Results

Specificity of Anti-Calpain I IgG and Anti-Calpain II IgG. The specificity of the affinity-purified anti-calpain I (or calpain II) 80K subunit IgG was studied with purified human erythrocyte calpain I, porcine erythrocyte calpain I, and porcine kidney calpain II as the test antigens. Each antigen protein, composed of 80K subunits and 30K subunits, was gel electrophoresed and then transferred to a nitrocellulose membrane. As shown in Figure 1b, anti-calpain I 80K subunit IgG binds to the 80K subunit of human erythrocyte calpain I but not to the 80K subunit of porcine kidney calpain II or to the 30K subunits of the two calpains. This IgG binds equally well to the 80K subunit of porcine erythrocyte calpain I as to that of human erythrocytes (lane B). When the anti-calpain II 80K subunit IgG was tested, monospecific binding to porcine kidney calpain II 80K subunit was also noted (Figure 1c).

Detection of Calpain by Immunoelectrophoretic Blotting. Figure 2a shows a Coomassie blue stained SDS–polyacrylamide gel to which several different samples of human erythrocyte membranes and the extracts therefrom were applied. Figure 2b shows the corresponding blots on which the localization of proteins was done with calpain I monospecific IgG. Calpain I was found in the cytosol, membranes, Triton membrane skeletons, and Triton extracts (lanes A and C–E). In the final washing (lane B), calpain I was not detected at all, providing evidence that the membranes used were devoid of buffer-extractable calpain I. Since the data shown in Figure 2 were found to be reproducible in at least 10 experiments, the levels of calpain I in various samples used were estimated from the apparent density of the blots and the amount of proteins applied. Thus, membrane-associated calpain I was approximately 2% of the cytosol enzyme. The bulk of this enzyme was extracted with 1% Triton X-100, while a minor portion still remained in Triton membrane skeletons. In addition, 1% Triton extracts, after removal of Triton X-100 with Bio-Beads SM 2, showed caseinolytic activity in the presence of 1 mM Ca^{2+} and cysteine. Such calpain activity was not detected in the extracts when 0.1% Triton X-100 was employed in the place of 1% Triton (data not shown). When calpain II monospecific IgG was used, there was no binding to any of the membrane fractions tested (Figure 2c).

Autodigestion of Membranes. Erythrocyte membranes were autodigested in the presence of 0.1 mM $CaCl_2$ and 5 mM cysteine. The digests were analyzed by SDS–polyacrylamide gel electrophoresis. Inside out vesicles were used to ensure easier contact of Ca^{2+} added to the medium with calpain I, which was assumed to be localized close to the inner surface of the membranes. The results obtained are shown in Figure 3. In no case was there evidence for the aggregation which would cause an apparent loss of some protein bands (Lorand et al., 1976). In the presence of Ca^{2+}, band 4.1 was preferentially degraded, forming low molecular weight peptides. While the breakdown of band 4.1 seemed to reach a plateau within 30 min (lanes B and C), the degradation of band 3 became detectable only upon longer incubation up to 5 h. There was also a degradative product below band 2 that was further degraded upon longer incubation. The calpain activity was inhibited by leupeptin (lane F), but not by calpastatin added to the medium (lane E). In a separate experiment, the caseinolytic activity of Triton extracts was found to be inhibited by calpastatin as well as leupeptin.

Discussion

We have recently succeeded in establishing an efficient method for isolating calpains (Yoshimura et al., 1983; Hatanaka et al., 1983), which enabled us to raise antisera against calpain I and against calpain II, respectively, yielding both anti-calpain I IgG and anti-calpain II IgG (Sasaki et al., 1983). Enzyme-linked immunosorbent assays using these IgG proteins

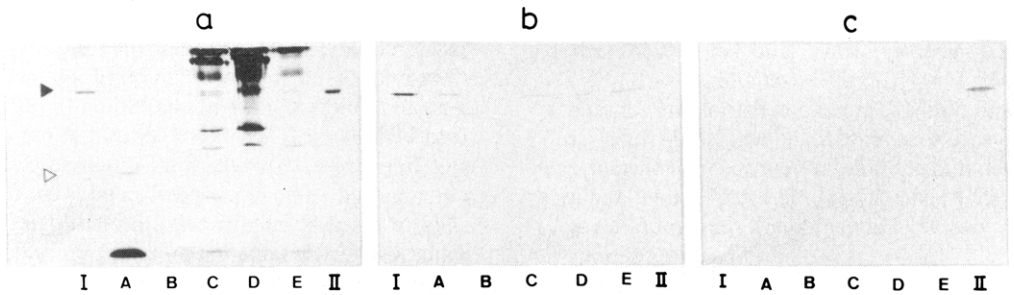

FIGURE 2: Detection of calpain by immunoelectrophoretic blotting. To lane I was applied 3 μg of purified human erythrocyte calpain I and to lane II 3 μg of porcine kidney calpain II. Lanes A–E are for the fractionated erythrocyte proteins as described under Experimental Precedures: lane A, erythrocyte cytosol (22.7 μg of protein); lane B, final washing (2.7 μg of protein); lane C, membranes (15.6 μg of protein); lane D, Triton membrane skeletons (24.7 μg of protein); lane E, Triton extracts (6.4 μg of protein). Part a shows the Coomassie blue stained SDS–polyacrylamide gel (12.0%). The proteins on gels were transferred to the nitrocellulose membranes and were depicted by the binding with affinity-purified anti-human erythrocyte calpain I IgG (b) or anti-porcine kidney calpain II IgG (c) using horseradish peroxidase conjugated goat anti-rabbit IgG. Closed arrowhead indicates the position of 80K subunit, and open arrowhead indicates the position of 30K subunit.

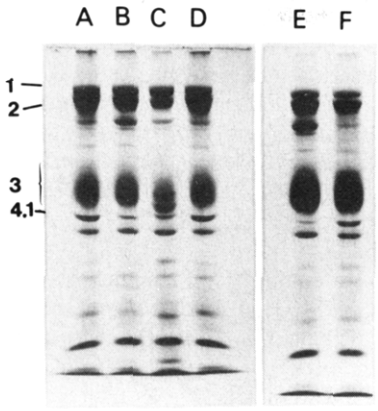

FIGURE 3: Autodigestion of erythrocyte membranes. Inside out vesicles were incubated in the presence of 0.1 mM $CaCl_2$ and 5 mM cysteine unless otherwise indicated, and the digests were analyzed by electrophoresis on SDS–polyacrylamide gel (7.5%). Approximately 100 μg of protein was applied to each lane. The gel was stained with Coomasie blue. Lane A, no incubation; lane B, incubation for 30 min; lane C, incubation for 5 h; lane D, incubation for 1 h in the absence of $CaCl_2$; lane E, pretreatment with human erythrocyte calpastatin for 10 min and then incubation for 1 h; lane F, pretreatment with 0.1 mM leupeptin and then incubated for 1 h.

revealed cross-reactivity to various extents between calpain I and calpain II, each either as a whole enzyme or after being resolved into its 80K and 30K subunits (Sasaki et al., 1983). It was further shown that the 80K subunits are immunologically dissimilar between calpain I and calpain II, whereas the 30K subunits are very similar (Wheelock, 1982; Sasaki et al., 1983). Therefore, to obtain a monospecific antibody for calpain I, or for calpain II, one should purify the antibody so as to attain monospecificity for the respective 80K subunits. Starting from the IgG proteins against the whole enzymes and using two steps of affinity chromatography on calpain I 80K subunit–Sepharose and calpain II 80K subunit–Sepharose, we were able, for the first time, to isolate an IgG fraction monospecific either for calpain I or for calpain II (Figure 1).

The immunoelectrophoretic blotting study with such a monospecific IgG preparation provided evidence for membrane-associated calpain I in human erythroctyes (Figure 2). The proportion of such associated enzyme to the total amount of calpain I in erythrocytes was small (2% as estimated), but the presence of the associated species was distinct, as shown in Figure 1, and was found to be constantly reproducible in a large number of experiments. It is unlikely that calpain I detected in erythrocyte membranes is a mere contaminant of the abundant cytosolic enzyme, because the membrane preparation used did not release any trace of calpain I unless it was extracted with the buffer containing 1% Triton X-100 (Figure 2b, lanes B and E). The membrane association of calpain I was found to be so strong that a considerable portion of the enzymes remained even after the extraction with 1% Triton X-100 (lane D) and that no caseinolytic activity could be measured in 0.1% Triton X-100 extracts of the membranes (data not shown). The caseinolytic activity in 1% Triton X-100 extracts became measurable after the detergent had been removed. This was different from the situation found for the cytosol, in which calpain activity was latent owing to the presence of a large excess of an endogenous inhibitor, calpastatin (Murakami et al., 1981).

The results shown in Figure 2c clearly indicate that no calpain II was detected in erythrocyte cytosol and in membranes. Since the IgG (anti-calpain II 80K subunit) used here was raised against porcine calpain, the question arises whether the observed lack of detection of calpain II in human erythrocytes could be due to species specificity. However, the previous report from this laboratory (Sasaki et al., 1983) has shown almost equal cross-reactivity of anti-calpain (I or II) IgG with human, porcine, and rat calpains as demonstrated by enzyme-linked immunosorbent assays. Also as shown in Figure 1b, IgG (anti-human calpain I 80K subunit) reacted equally well with human and porcine calpain I.

The action of calpain I associated with the membranes is considered to be responsible for the breakdown of band 4.1 observed in autodigestion of human erythrocyte membranes, because the digestion was found to be entirely Ca^{2+} dependent and inhibited by leupeptin (Figure 3, lanes D and F). The Ca^{2+} requirement and leupeptin sensitivity are two important criteria for the action of calpain (Murachi et al., 1981b). That the autodigestion was not inhibited by calpastatin (Figure 3, lane E) does not mean that the responsible enzyme is something else than calpain, but it means that a large molecular weight compound like calpastatin (~280 000) (Takano & Murachi, 1982) would not be able to reach inside the membrane layer in which calpain molecules exist. Band 4.1 was shown to be resolved into two components by SDS–polyacrylamide gel electrophoresis with a discontinuous buffer system (Mueller & Morrison, 1977), which later proved to be mutually related in primary structures (Goodman et al., 1982). Since we could not attain such resolutions, it is not known whether one of the components of band 4.1 is more susceptible to calpain than the other or whether the two bands are equally susceptible. The observed incomplete digestion of band 4.1 even upon longer incubation (Figure 3, lane C), however, may suggest the more preferential digestion occurring with either one of the two components of band 4.1.

It is well-known that spectrin, actin, and band 4.1 are the major erythrocyte cytoskeletons (Branton et al., 1981). Moreover, band 4.1 has been shown to modulate the association between spectrin and actin by making them thixotropic (Cohen & Korsgren, 1980). Thus, calpain, through its action on band 4.1, is supposed to have an important role in regulating deformability of erythrocyte membranes. The digestion of band 4.1 was earlier observed by incubating in vitro the membrane preparations with isolated cytosol calpain I (Murakami et al., 1981). However, it is unclear whether the cytosol calpain I can actually act on membrane proteins in vivo because of its latency due to coexisting calpastatin. In contrast, membrane-associated calpain I is not latent, and surprisingly it is not inhibited by calpastatin as far as the autodigestion is concerned (Figure 3, lane E). In this regard, membrane-associated calpain may be functionally significant. However, it is not yet known whether membrane-associated calpain is a constituent of the membranes distinct from the cytosol enzyme or whether it is a portion of the cytosol enzyme which is firmly bound to the membranes under certain conditions, as was suggested earlier (Allen & Cadman, 1979). Further investigation will be needed to discriminate between these two possibilities.

The calpain activity has also been detected in association with intermediate filament preparation (Nelson & Traub, 1981; Ishizaki et al., 1983). Intermediate filament proteins including neurofilaments, desmin, and vimentin were shown to be extremely susceptible to Ca^{2+}-dependent proteolysis, and the disassembly of intermediate filaments was supposed to be regulated by such proteolysis (Lazarides, 1980). Regulation of polymerization was also shown by using cytosol calpain preparations with microtubules (Sandoval & Weber, 1978) and with actin filaments (Davies et al., 1978; Truglia & Stracher, 1981). It is thus postulated that the generalized role

of calpain is to regulate disassembly of the cytoskeletal structures, although the target cytoskeletal proteins may differ from one type of cell to the other and the resultant physiological phenomena may also differ. It remains to be seen if calpain, acting on different cytoskeletal proteins in different cells, exerts such seemingly diverse reactions through the same specificity common to most, if not all, of the substrate polypeptides or through each different specificity with each one of the different substrates involved.

Acknowledgments

We thank Naomi Kawaguchi for technical assistance. The skillful secretarial assistance of Yoko Sugihara, Katsuko Sano, and Kyoko Ikeda is gratefully acknowledged.

Registry No. Calpain, 78990-62-2.

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