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Proteolytic Modification of Calcium-Dependent Protease 1 in Erythrocytes Treated with Ionomycin and Calcium†

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ABSTRACT: In vitro, limited proteolytic cleavage of the subunits of the purified calcium-dependent proteases [also known as calpains (EC 3.4.22.17) or calcium-activated neutral proteinases (CANPs)] appears to be required for enzyme activity. It has not yet been demonstrated if similar processing of the protease subunits occurs in vivo. To directly assess proteolytic modification of these proteases in cells, we have measured the loss of the proenzyme form of the regulatory subunit (a 26-kDa protein) and/or the appearance of the modified regulatory subunit (a 17-kDa protein) by densitometric analysis of immunoblots. In rat erythrocytes, proteolytic modification of the endogenous calcium-dependent protease (calcium-dependent protease 1, μCANP) occurs in vivo in response to ionomycin and calcium. The extent of enzyme modification was dependent on time, ionomycin concentration, and calcium concentration, suggesting that in this cellular model Ca^{2+} regulates proteolytic modification of the enzyme.

Recently it has become clear that the two intracellular calcium-dependent proteases [also known as calpains or calcium-activated neutral protease (CANP, EC 3.4.22.17)] are inactive proenzymes (DeMartino et al., 1986; Suzuki et al., 1987; Pontremoli & Melloni, 1986). Each proenzyme is a heterodimer composed of a unique catalytic subunit (80 kDa) and a common regulatory subunit (26 kDa). Studies of the purified proteases have demonstrated proteolytic processing of each of the protease subunits under conditions where proteolytic activity is measured (DeMartino et al., 1986; Suzuki, 1987; Suzuki et al., 1987; Mellgren et al., 1982; Coolican et al., 1986; Pontremoli & Melloni, 1986; Inomata et al., 1985; Imajoh et al., 1986). The autoproteolyzed enzymes are active against protein substrates and have reduced calcium requirements for this activity (Suzuki et al., 1987; Coolican et al., 1984; Coolican & Hathaway, 1986; Mellgren et al., 1982; DeMartino et al., 1986; Inomata et al., 1985). Proteolysis of the larger catalytic subunit is sometimes (Croall & DeMartino, 1984; Inomata et al., 1984, 1985; Pontremoli & Melloni, 1986; Samis et al., 1987) but not always detectable by denaturing gel electrophoresis (Inomata et al., 1984, 1985; DeMartino et al., 1986; Suzuki et al., 1987; Mellgren et al., 1982) depending on the type and source of calcium-dependent protease. Modification of the regulatory subunit of each protease changes the 26-kDa protein, through several intermediates, to a 17-kDa form (Mellgren et al., 1982; Coolican et al., 1986; DeMartino et al., 1986; Suzuki, 1987; Suzuki et al., 1987). There is currently some controversy as to the exact correlations between specific cleavage events and protease function(s) (Coolican & Hathaway, 1986; Mellgren et al., 1982; Suzuki et al., 1985, 1987; DeMartino et al., 1986). However, the enzyme proteins are proteolytically modified if substrates are hydrolyzed or if the enzymes are exposed to conditions appropriate for enzyme activity in vitro. Because proteolytic modification of the enzyme subunits is a unidirectional process, the presence of the modified enzyme subunits provides evidence that the enzymes are, or have been, active. If similar proteolytic modification occurs in vivo, the change in protease composition would provide direct evidence for enzyme acti-

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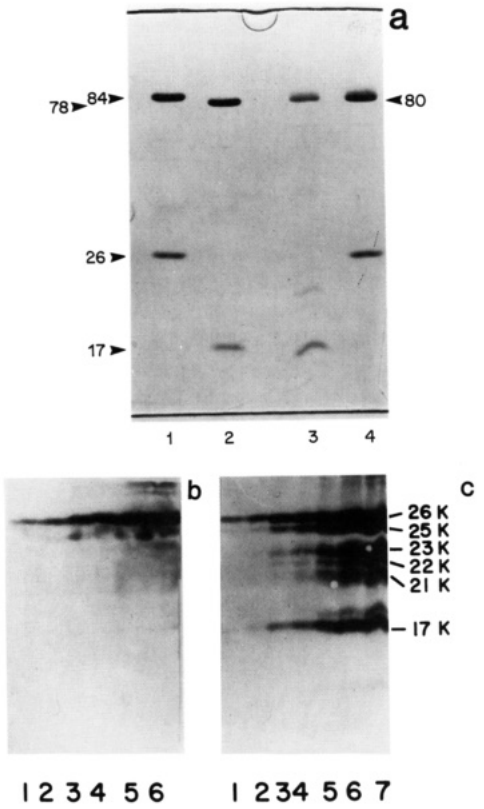

FIGURE 1: Electrophoretic separation and immunostaining of the native and autoproteolyzed calcium-dependent proteases. (Panel a) SDS–PAGE of native and autoproteolyzed calcium-dependent proteases 1 and 2 (CANP-1 and CANP-2) stained with Coomassie blue. CANP-1 (lane 1) and CANP-2 (lane 4) were purified from bovine heart and electrophoresed as described under Materials and Methods. Each native protease was incubated for 5 min under conditions known to allow autoproteolytic modification of each enzyme: 5 mM $CaCl_2$, 1 mM EDTA, and 1 mM EGTA at 4 °C for CANP-1; 7 mM $CaCl_2$ and 1 mM EGTA at 25 °C for CANP-2. Samples were then heated in SDS–PAGE sample buffer and electrophoresed (modified CANP-1, lane 2; modified CANP-2, lane 3). Each lane was loaded with 10 μg of protein. (Panels b and c) Immunoblot staining of the native and autoproteolyzed regulatory subunit of calcium-dependent protease 2. Native CANP-2 (panel b, lanes 1–6) or partially autoproteolyzed CANP-2 (1 min, 25 °C, 7 mM $CaCl_2$; panel c, lanes 1–7) was treated with SDS–PAGE sample buffer. Various amounts of CANP-2 were mixed with 100 μg of SDS-treated proteins (heart extract prepared as described under Materials and Methods) electrophoresed, and immunoblotted. Only the lower portion of the blots, containing the regulatory subunit, is shown. The amount of 26-kDa subunit in each lane was 5, 10, 50, 100, 250, or 500 ng, respectively, for lanes 1–6 of each panel; panel c, lane 7, contained 1 μg of 26 kDa.

vation. In this report, we describe an immunoblot procedure to measure proteolytic modification of the regulatory subunit of each calcium-dependent protease and demonstrate proteolytic processing of this subunit of the endogenous calcium-dependent protease in rat erythrocytes treated with ionomycin and calcium.

Materials and Methods

Preparation of Enzymes and Antibodies. Both calcium-dependent proteases were purified from bovine cardiac muscle as described previously (Croall & DeMartino, 1984). The bovine proteases are composed of 84-kDa/26-kDa (calcium-dependent protease 1) or 80-kDa/26-kDa (calcium-dependent protease 2) proteins (Figure 1a). In all mammalian tissues, the calcium-dependent proteases are heterodimers composed of a catalytic subunit (~80 kDa) and a regulatory subunit (~30 kDa). The exact size of each subunit varies somewhat species to species (Pontremoli & Melloni, 1986; Goll et al., 1985; DeMartino & Croall, 1983). In this paper, I refer to the unmodified regulatory subunit as 26 kDa. Polyclonal antisera were raised in New Zealand white rabbits as detailed elsewhere (Beckerle et al., 1987). Antisera raised against each native protease were characterized by the indirect immunoblot techniques described below. Antibodies used throughout this study were raised against the calcium-dependent protease 2 isolated from bovine heart. This serum recognizes the 26-kDa subunit of both enzymes and the 80-kDa, catalytic subunit of the antigen and cross-reacts to a limited degree with the catalytic subunit of calcium-dependent protease 1. Using the antibody raised against the type 2 enzyme to probe for the type 1 regulatory subunit minimizes the possibility of detecting fragments of any degraded catalytic subunit.

To test the sensitivity and specificity of the antisera, an extract was prepared from bovine heart as described previously (Croall & DeMartino, 1984). To remove both endogenous calcium-dependent proteases, NaCl was added to 0.5 M, and the extract was passed through phenyl-Sepharose equilibrated with 50 mM Tris, pH 7.5 at 4 °C, 0.5 M NaCl, 2 mM EDTA, 2 mM EGTA, and 5 mM 2-mercaptoethanol. The flow-through fraction (depleted of calcium-dependent proteases) was dialyzed against buffer without NaCl, concentrated, and treated with SDS–PAGE sample buffer. This protein mixture was added to known amounts of purified proteases for the immunoblots shown in Figure 1b,c.

Immunoblot Analysis of Calcium-Dependent Protease Subunits. Purified proteases or cellular proteins prepared as described below were displayed by gel electrophoresis on 12% acrylamide gels [37.5:1 acrylamide/*N*,*N*′-methylenebis-(acrylamide)] in the presence of sodium dodecyl sulfate (SDS–PAGE) using Tris–glycine buffers. Prestained molecular weight markers were run on each gel to allow immediate visualization of the markers after transfer of proteins to nitrocellulose. Proteins were transferred electrophoretically to nitrocellulose paper (Towbin et al., 1979) for 4 h at 170 mA, and for an additional 12–14 h at 80 mA. After transfer, the filters were cut at a molecular weight of 35 000–40 000 to allow separate incubations of filters containing the small regulatory subunits (17–28 kDa) of the protease and the blot that contained the catalytic subunits (80–84 kDa). Prior to incubation with anti-calcium-dependent protease 2 serum, filters were blocked for 1 h in 3% gelatin (or 5% nonfat dry milk) in Tris-buffered saline (20 mM Tris, pH 7.5, and 0.5 M NaCl; TBS). Filters were incubated with a 1:300 dilution of primary antisera in 1% gelatin in TBS and 0.05% Tween 20 for 3 h at 20 °C. After blots were washed in TBS containing 0.05% Tween 20, goat anti-rabbit IgG conjugated to alkaline phosphate (Bio-Rad) was added (1:3000) and incubated for 1 h. Immunoreactive material was visualized with alkaline phosphatase substrates, 5-bromo-4-chloro-3-indolyl phosphate and toluidine *p*-nitroblue tetrazolium chloride. Figures 1 and 2 document the sensitivity and specificity of the alkaline phosphatase linked immunoblot analysis of the small subunit of the native and autoproteolyzed forms of each calcium-dependent protease. Quantification of stained bands was accomplished with an LKB-Ultroscan laser densitometer. The intensity of immunostaining of the 17-kDa band relative to the 26-kDa band (Figures 1c and 2a) demonstrated that the available polyclonal sera reacts with epitopes throughout this protein. To estimate the degree of proteolytic modification of the regulatory subunit, the following calculations were used:

(1) The equation (immunoreactive area 17 kDa)(1.52)/[(immunoreactive area 17 kDa)(1.52) + (immunoreactive area 26 kDa)] was used where the factor 1.52 is the ratio of mo-

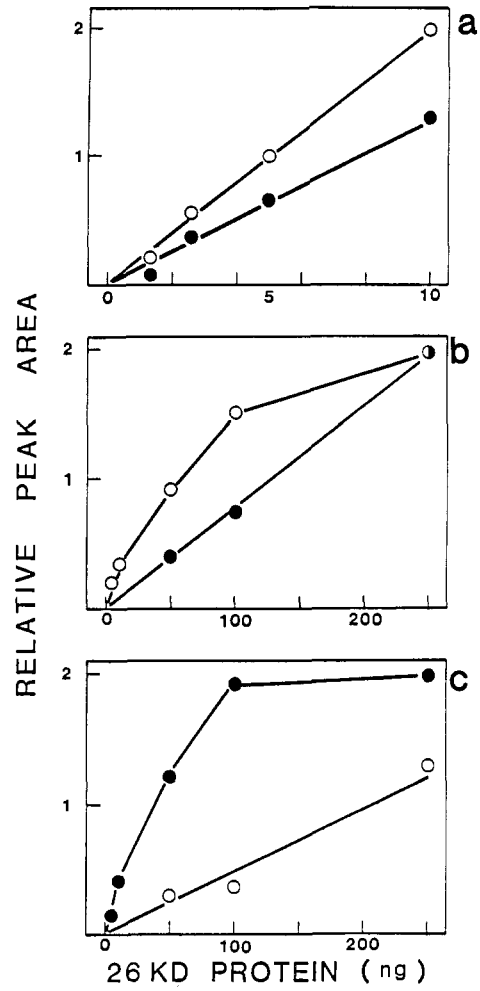

FIGURE 2: Immunoblot analysis of the 26-kDa subunit of the calcium-dependent proteases and its 17-kDa fragment: sensitivity of detection using alkaline phosphatase linked goat anti-rabbit IgG. Immunoblots were prepared and probed as described under Materials and Methods. In each panel, the densitometric area is plotted relative to the amount of total 26-kDa protein present in the sample. Densitometric analysis of a representative blot of untreated (○) and autoproteolyzed (●) calcium-dependent protease 1 is shown in panel a. Densitometric analysis of blots of autoproteolyzed calcium-dependent protease 2 is shown in panels b and c. Autoproteolysis occurred for 1 min (panel b) or 5 min (panel c) prior to heating the enzyme in SDS–PAGE sample buffer. These blots were scanned for both the 26-kDa (○) and 17-kDa (●) proteins. To compare the areas of 17 kDa and 26 kDa within panel b or c, the relative area for 17 kDa is half that for 26 kDa (panel b) or twice that of 26 kDa (panel c).

lecular weights of the 26-kDa subunit to the 17-kDa subunit. Because some epitopes are lost during proteolytic cleavage, this should be more representative of the molar ratios of the two proteins than the uncorrected immunoreactive area (ira).

(2) If only the loss of the 26-kDa protein was assessed, without monitoring the relative amounts of the 26-kDa/17-kDa proteins, the calculation was used:

$$\frac{(\text{ira 80 kDa,control}/\text{ira 80 kDa,exptl})(\text{ira 26 kDa,exptl})}{\text{ira 26 kDa,control}}$$

Measuring the relative immunoreactivity of the catalytic subunits (80 kDa) allows correction for differences in yields from the ion-exchange column or blotting efficiency; the molar ratio of 80 kDa/26 kDa within a sample will increase as 26 kDa is proteolyzed.

Loading Erythrocyte Ghosts with Casein and Calcium-Dependent Protease 2. Outdated human red blood cells were washed and loaded with exogenous proteins according to the method of Rechsteiner (1975). [*methyl*-^{14}C]Casein was prepared as previously described (Dottavio-Martin & Ravel, 1978) and had a specific activity of 10 000 dpm of ^{14}C/μg of casein. [*methyl*-^{14}C]Casein and calcium-dependent protease 2 were dialyzed separately against 5 mM Tris, pH 7.5 at 4 °C, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Preswollen erythrocytes were lysed at 4 °C by addition of the hypotonic solutions of dialyzed protease (0.4 mg/mL) and radiolabeled casein (5 mg/mL). After 3–4 min on ice, 0.1 volume of 1.5 M NaCl in 0.1 M sodium phosphate, pH 7.5, was added, and erythrocyte ghosts were incubated at 37 °C for 1 h to allow resealing. Loaded ghosts were collected by centrifugation and washed 5 times with 5 mM Tris, pH 7.5, and 150 mM NaCl prior to the initiation of each experiment.

Rat Erythrocytes. Rat blood was collected from anesthetized rats by cardiac puncture. Blood was collected into syringes containing 112 mM trisodium citrate and 6 mM EDTA as anticoagulant. After centrifugation, cells were resuspended in 0.15 M NaCl and passed through a microcrystalline cellulose/α-crystalline cellulose (1:1) column as described by Beutler et al. (1976) to remove leukocytes and platelets. Erythrocytes were washed 2–3 times in sodium acetate/sodium chloride, pH 5.6 (Kosower et al., 1983). Immediately before use, cells were sedimented, resuspended, and washed in 5 mM Tris, pH 7.5, 100 mM KCl, 60 mM NaCl, 10 mM glucose, and 2 mM $MgCl_2$. Experiments were performed at 37 °C in the presence of calcium chloride and ionomycin or ethanol as detailed in the figure legends. Ionophores such as ionomycin result in increased flux of Ca^{2+} across the membrane and an increase in $[Ca^{2+}]_i$, but because of the functioning Ca^{2+}-ATPase within erythrocyte membranes, $[Ca^{2+}]_i$ does not reach extracellular levels (Calviello et al., 1987; Carafoli, 1987). Incubations were terminated by addition of isotonic EGTA and centrifugation at 600*g* for 10 min. Cell pellets were lysed in 10 volumes of 5 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, and 5 mM 2-mercaptoethanol (lysis buffer). Lysates were separated from ghost membranes and unlysed red cells by centrifugation at 20000*g* for 20 min. The pellet was resuspended in 10 volumes of lysis buffer and recentrifuged. The pooled lysates were incubated with DEAE-Sephacel (1.5 mL) that had been preequilibrated with lysis buffer. The resin was stirred with lysate intermittently over 20–30 min. After the resin settled, unbound protein was decanted; the resin was packed into a small column and washed with 5–10 column volumes of lysis buffer prior to elution of bound proteins with 0.5 M NaCl in lysis buffer. Eluted proteins were dialyzed to lower the NaCl concentration, and samples were concentrated (Centricon 10 microconcentrator) prior to protein determination (Bradford, 1979) and heating with SDS sample buffer.

RESULTS

Proteolytic Modification of Calcium-Dependent Proteases 1 and 2. The purified calcium-dependent proteases are autoproteolytically modified under conditions required for their activity against protein substrates. This proteolytic modification may be an activation mechanism which is necessary for calcium-dependent proteolysis of other proteins (Coolican et al., 1986; DeMartino et al., 1986; Mellgren et al., 1982; Pontremoli & Melloni, 1986; Suzuki et al., 1987; Imajoh et al., 1986; Inomata et al., 1985). The proteolytically modified proteases are not, however, isolated to any significant extent during enzyme purification in the presence of chelators (Croall & DeMartino, 1987; DeMartino et al., 1986). It has not yet been documented that proteolytic processing of the enzymes occurs either in crude extracts or in intact cells. To establish

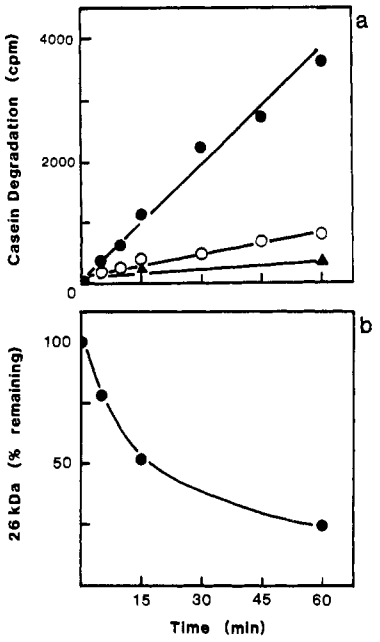

FIGURE 3: Activation of CANP-2 and hydrolysis of casein in CANP-2, [*methyl*-^{14}C]casein-loaded erythrocyte ghosts. (Panel a) Erythrocytes were loaded and resealed as described under Materials and Methods. Loaded ghosts were suspended in 5 mM Tris, pH 7.5, 150 mM NaCl and 0.5 mM dithiothreitol and incubated at 25 °C with 1 mM $CaCl_2$ in the presence (●) or absence (○) of 1 μM ionomycin for the times shown. Ghosts loaded with [^{14}C]casein but without exogenous protease (▲) were incubated with calcium and the ionophore as a control for any retained endogenous caseinolytic activity. At various times, aliquots of the incubation were mixed with 5 mM Tris, pH 8.0, 2 mM EDTA, and 2 mM EGTA and immediately made 10% in trichloroacetic acid. After centrifugation, the acid-soluble ^{14}C was measured by liquid scintillation counting. (Panel b) Aliquots of the incubation mixtures described for (a) were alternatively treated with SDS sample buffer for analysis by SDS–PAGE and immunoblotting. Immunoreactive bands were scanned and plotted as percent 26-kDa protein remaining relative to controls (no calcium added). No immunoreactive CANP was detected in mock-loaded cells, and the levels of immunoreactive 26-kDa protein remained constant in cells not treated with ionophore or calcium (not shown).

that proteolytic modification of each calcium-dependent protease also occurs in the presence of cellular proteins, including endogenous substrates, two types of experiments were done. First, erythrocyte ghosts were loaded with calcium-dependent protease 2 and radiolabeled casein. We had previously shown that proteolysis of membrane skeletal proteins occurs when such resealed ghosts are incubated with ionophore and calcium (Croall et al., 1986). Autoproteolytic cleavage of the 26-kDa subunit of calcium-dependent protease 2 occurred concomitantly with proteolysis of the radiolabeled casein after introduction of calcium by ionomycin (1 μM) (Figure 3).

Second, extracts were prepared from rat erythrocytes as described under Materials and Methods. Immunoblot analysis of an extract before and after the addition of calcium chloride demonstrated proteolytic processing of the regulatory subunit of endogenous calcium-dependent protease 1, decreasing the 26-kDa protein and increasing its 17-kDa fragment (data not shown). Thus, even in the presence of numerous proteins susceptible to calcium-dependent proteolysis (Croall et al., 1986; Pontremoli & Melloni, 1986), proteolytic modification of the rat protease occurs as it does with the purified bovine enzymes in the absence of substrates.

Proteolytic Modification of the Endogenous Calcium-Dependent Protease 1 of Rat Erythrocytes in Situ. Rat erythrocytes were selected as a model to examine proteolysis of calcium-dependent protease 1 in intact cells. Erythrocytes contain only the type 1 calcium-dependent protease, and proteolysis of the membrane skeletal proteins is reported to be rapid in these cells after exposure to ionomycin and calcium (Kosower et al., 1983). We confirmed the observation of Kosower et al. (Kosower et al., 1983; Glaser & Kosower, 1986) by demonstrating extensive proteolysis of membrane skeleton proteins in erythrocytes treated with ionomycin and calcium (Figure 4a). The abundance of hemoglobin and membrane skeletal proteins relative to the protease prevented immunoblot analysis of whole cell extracts. Thus, the calcium-dependent protease 1 was partially purified from erythrocyte lysates as described under Materials and Methods. Experiments with the purified proteases in their unautolyzed and proteolytically modified forms demonstrated that both forms of each protease bind to and elute from the ion-exchange resin using the conditions described (data not shown).

If increased intracellular calcium concentration is sufficient to stimulate proteolytic modification of the calcium-dependent protease in rat erythrocytes, we expected that the decrease in the amount of the 26-kDa subunit (or the increase in the amount of the 17-kDa fragment) would be proportional to increasing [Ca^{2+}]. To alter cellular calcium homeostasis, we increased either the time of incubation in the presence of ionophore and calcium, the ionophore concentration, or the extracellular calcium concentration. Results of these experiments are shown in Figures 4–6. When both extracellular calcium and ionophore are present, the 26-kDa subunit of calcium-dependent protease 1 was converted to a 17-kDa protein. The extent of conversion of the regulatory subunit correlated with increasing time (Figures 4b and 5), increasing ionophore concentration at a fixed calcium concentration, and increasing calcium concentration at a fixed ionophore concentration (Figure 6). Similar results were obtained by using polyclonal sera from two different rabbits (Figure 5A) or affinity-purified antibodies (data not shown) that were isolated by adsorption to and elution from nitrocellulose-immobilized, SDS–PAGE-purified protease subunits (Olmsted, 1983; Beckerle et al., 1987). When the erythrocyte extracts were probed with affinity-purified antibodies or whole sera, the amount of enzyme modification measured was similar although the background immunoreactivity was significantly reduced with affinity-purified antisera. If the antisera were preadsorbed against the purified calcium-dependent protease 2 subunits, immunostaining of the 80-kDa/26-kDa and 17-kDa proteins in the erythrocyte samples was reduced 85–95% (data not shown). Antisera specific for the catalytic subunit of calcium-dependent protease 1 did not stain the 26-kDa or 17-kDa proteins in the erythrocyte lysate samples (data not shown). This further substantiates that the immunoreactivity measured results from the 26-kDa regulatory subunit and its 17-kDa fragment and not breakdown products of the catalytic subunit. Under conditions where proteolysis of the protease 26-kDa subunit was demonstrated, we also observed proteolysis of membrane skeleton proteins (as shown in Figure 4a) and the appearance of several membrane skeleton proteins (or protein fragments) in these cell lysates (data not shown).

Discussion

We have shown that the proenzyme form of the calcium-dependent protease within rat erythrocytes is converted to a proteolyzed, active form of the enzyme in cells treated with ionomycin in the presence of calcium. Previously, proteolysis of the regulatory subunit had only been described with the purified enzymes in vitro. It seems likely that the proteolytic processing, apparently required for activation of the proenzyme, is autocatalytic in vivo, as it is in vitro, although this

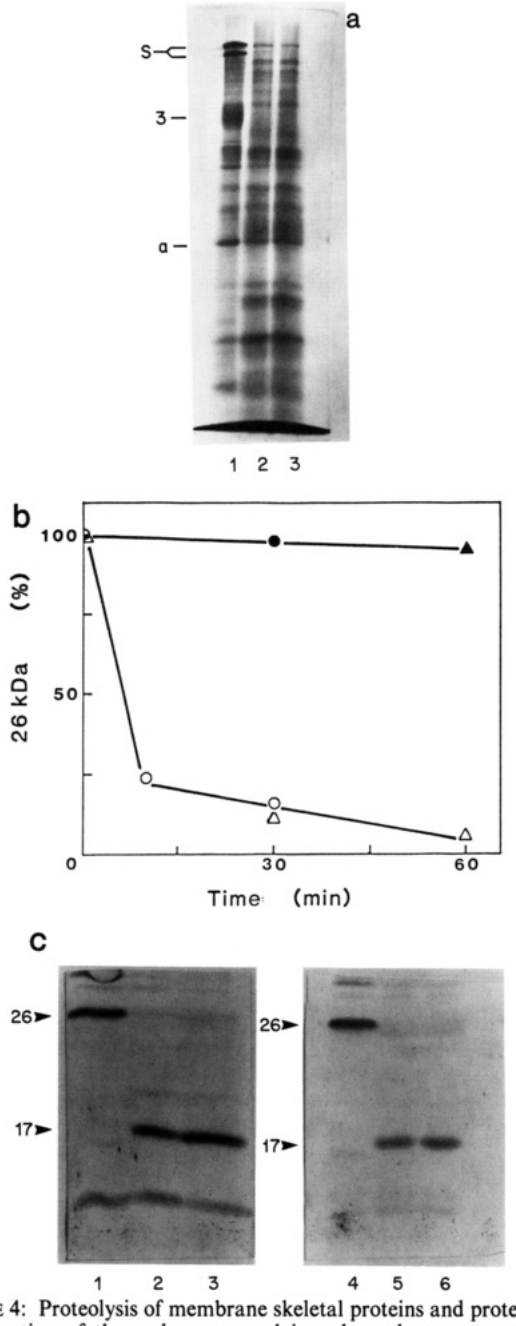

FIGURE 4: Proteolysis of membrane skeletal proteins and proteolytic modification of the endogenous calcium-dependent protease 1 in erythrocytes treated with ionomycin and calcium. (Panel a) SDS–PAGE of erythrocyte ghost membranes. Rat erythrocytes were incubated (37 °C) with ionomycin (10 μM) in the presence of 0.5 mM $CaCl_2$ for 0, 30, or 60 min. At each time, EGTA was added to 1 mM, and cells were sedimented and subsequently lysed as described under Materials and Methods. After the ghosts were washed 5 times, the sedimented membranes were treated with SDS–PAGE sample buffer. The gel shown [10% acrylamide, 75:1 acrylamide/*N*,*N*-methylenebis(acryalmide)] was stained with Coomassie blue. Spectrins (S), band 3 (3), and actin (a) are designated as shown. (Panel b) Immunoblot analysis of erythrocyte calcium-dependent protease 1. Rat erythrocytes were incubated as in panel a with ionomycin (10 μM) for 0–60 min (○, △) or in the presence of ethanol (0.1%; ●, ▲). The cell lysates were prepared for and immunobloted as described under Materials and Methods. Densitometric analysis is shown for the immunoblots pictured (panel c) and the ethanol-treated controls (●, ▲; blot not shown). Data are plotted as percent 26-kDa subunit remaining relative to the controls. (Panel c) Lanes 1 and 4 show lysates from cells incubated with 10 μM ionomycin for 30 min in the absence of $CaCl_2$. The other lysates were prepared from cells treated with ionomycin and calcium for 30 min (lane 2) or 60 min (lane 3), and for 10 min (lane 5) or 30 min (lane 6). The low molecular weight band stained in lanes 1–3 was an artifact of this particular lysate and was not included in the densitometric analysis.

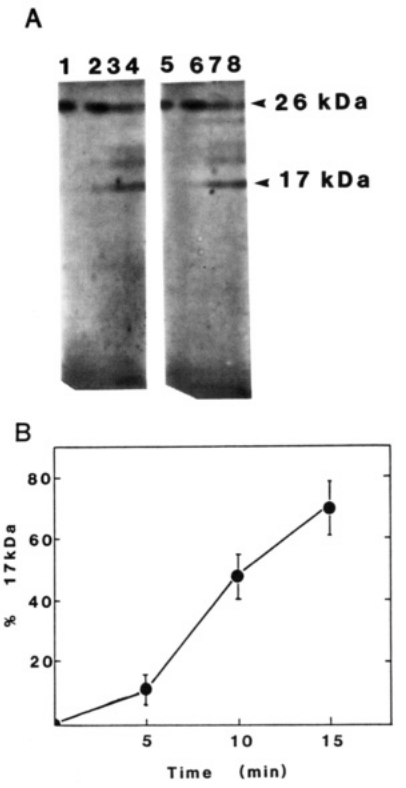

FIGURE 5: Immunoblot analysis of rat erythrocyte calcium-dependent protease 1. (Panel A) Immunoblot of rat erythrocyte lysates stained with two different polyclonal antisera raised against CANP-2. Erythrocytes were incubated at 37 °C in the presence of 1 mM $CaCl_2$ with either 5 μM ionomycin (lanes 2–4 and 6–8) or ethanol (0.1%) (lanes 1 and 5). Incubation was for 5 min (lanes 2, 6), 10 min (lane 3, 7), or 15 min (lanes 1, 4, 5, 8). Samples were prepared and immunoblotted as described under Materials and Methods. The blots shown are identical except that different polyclonal antisera against CANP-2 were used to probe each. Lanes 1 and 5 contained 35 μg of protein; lanes 2–4 and 6–8 contained 50 μg of protein. (Panel B) Densitometric analysis of the 26-kDa and 17-kDa proteins. The samples described and shown in panel A were electrophoresed, blotted, and probed numerous times using each of the two antisera, and also affinity-purified antisera. The densitometric analyses of these samples were combined for each time point, and standard deviations of the results were determined: $n = 10$ for t_5; $n = 22$ for t_{10} and t_{15}.

is not yet proven. The extent of enzyme modification is proportional to ionomycin and calcium concentration, suggesting that, in these cells, calcium (either concentration or frequency of transients) regulates enzyme activation in vivo. This is an important finding because there are many factors that modulate protease function in vitro and could potentially regulate enzyme activation in vivo (Croall & DeMartino, 1987; Mellgren, 1987; Pontremoli & Melloni, 1986; Suzuki et al., 1987). For example, in the presence of an excess of its inhibitor protein (calpastatin), the enzymes remain inactive irrespective of calcium concentration. Although the described immunoblot analysis will indicate that autoproteolysis of the enzyme has occurred, the amount of modified enzyme may not directly correlate with enzyme activity in vivo because of the two additional regulators of these proteases: the inhibitory protein and a stimulatory protein (DeMartino & Blumenthal, 1982). Comparison of enzyme modification in cells with different inhibitor to protease ratios (e.g., human versus rat erythrocytes) may provide important insights as to the role of the inhibitor protein in regulating calcium-dependent protease activity and/or activation.

The immunoblot analysis, as described, does not distinguish between the two calcium-dependent proteases. For erythrocytes, that have only type 1, and for platelets, that have pre-

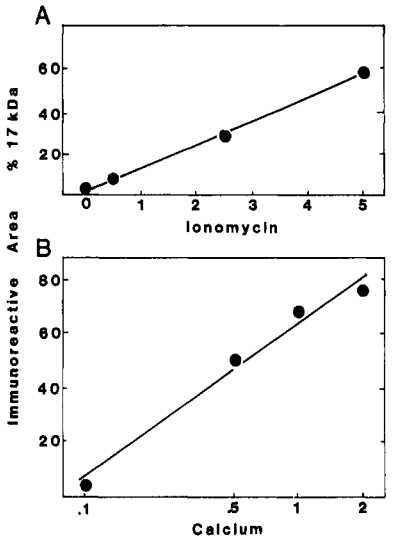

FIGURE 6: Effect of ionomycin and calcium concentration on proteolytic modification of CANP-1 in rat erythrocytes. (Panel A) Rat erythrocytes were incubated at 37 °C for 10 min in the presence of 0–5 μM ionomycin in the presence of 0.5 mM calcium chloride. Samples were prepared and analyzed by immunoblotting as described under Materials and Methods. Data are plotted as the densitometric area of the immunoreactive 17-kDa protein as a percentage of the total 17–26-kDa immunoreactive area to indicate the increase in the concentration of the autoproteolytically modified CANP-1. (Panel B) Experiments were done as in panel A except that cells were incubated with 2.5 μM ionomycin and the calcium chloride concentration was varied between 0.1 and 2.0 mM. Incubation time for the experiment shown was 10 min. Data shown in each panel are from multiple scans (3) of a single experiment and are representative of three such experiments; however, the extent of proteolytic modification of the enzyme for a given condition does vary for different batches of erythrocytes.

dominantly type 1 (Fox et al., 1985; Samis et al., 1987; D. E. Croall, unpublished observation), it is clear that the immunoreactive signal observed in cellular extracts is from the type 1 enzyme. In cells where each protease is present in similar amounts, it will be desirable to distinguish between the two proteases to analyze proteolytic modification of the individual enzymes. One available possibility is chromatography of cellular extracts on reactive red agarose which separates the two proteases (Clark et al., 1985). Preliminary results demonstrate equivalent recoveries of the autoproteolyzed and proenzyme forms of calcium-dependent protease 2 using this resin (unpublished observation). A second possibility is to examine proteolytic modification of the catalytic subunit of calcium-dependent protease 1 which produces a 78-kDa fragment that can be resolved from the proenzyme (84 kDa) by SDS–PAGE in some species (bovine and human, but not rat). Recently, two groups have described proteolysis of the type 1 84-kDa protein to its 78-kDa form in thrombin-activated, aggregated platelets (Samis et al., 1987; Okita et al., 1987). These results, as well as the demonstration of proteolysis of the protease regulatory subunit in ionomycin- or dibucaine-treated platelets (Croall, 1988), provide direct evidence for the activation of this enzyme during platelet aggregation (Fox et al., 1985).

Much circumstantial evidence suggests important functions for the calcium-dependent proteases in the cleavage of cytoskeletal and membrane skeletal proteins (Kosower et al., 1983; Fox et al., 1985; Nelson & Traub, 1982; Samis et al., 1987; Beckerle et al., 1987), protein kinase C (Kishimoto et al., 1983; Murray et al., 1987), and the receptors for EGF, PDGF, and other effectors (Cassel & Glaser, 1982; Ek & Heldin, 1986; Lynch et al., 1986). However, our lack of knowledge as to the endogenous targets of these proteases and the lack of specificity of available protease inhibitors have prevented direct demonstration of a role for calcium-dependent proteases in physiological proteolysis. The assay described in this paper will allow us to test the hypothesis that the activation of the calcium-dependent proteases is linked to Ca^{2+}-signaling events in other cell types. If we identify cellular conditions that increase proteolytic modification of the CANP enzymes, we may then be able to determine which of the proposed endogenous substrates are in fact physiological targets of this proteolytic system.

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Metal Ion Requirements for Sequence-Specific Endoribonuclease Activity of the *Tetrahymena* Ribozyme†

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ABSTRACT: A shortened form of the self-splicing intervening sequence RNA of *Tetrahymena thermophila* acts as an enzyme, catalyzing sequence-specific cleavage of RNA substrates. We have now examined the metal ion requirements of this reaction. Mg^{2+} and Mn^{2+} are the only metal ions that by themselves give RNA enzyme activity. Atomic absorption spectroscopy indicates that Zn, Cu, Co, and Fe are not present in amounts equimolar to the RNA enzyme and when added to reaction mixtures do not facilitate cleavage. Thus, these ions can be eliminated as cofactors for the reaction. While Ca^{2+} has no activity by itself, it alleviates a portion of the Mg^{2+} requirement; 1 mM Ca^{2+} reduces the Mg^{2+} optimum from 2 to 1 mM. These results, combined with studies of the reactivity of mixtures of metal ions, lead us to postulate that two classes of metal ion binding sites are required for catalysis. Class 1 sites have more activity with Mn^{2+} than with Mg^{2+}, with the other divalent ions and Na^+ and K^+ having no activity. It is not known if ions located at class 1 sites have specific structural roles or are directly involved in active-site chemistry. Class 2 sites, which are presumably structural, have an order of preference $Mg^{2+} \geq Ca^{2+} > Mn^{2+}$ and $Ca^{2+} > Sr^{2+} > Ba^{2+}$, with Zn^{2+}, Cu^{2+}, Co^{2+}, Na^+, and K^+ giving no detectable activity over the concentration range tested.

Some protein enzymes bind specific metal ions and use them for catalysis. Examples of metalloenzymes are carboxypeptidase A, carbonic anhydrase, alkaline phosphatase (Zn^{2+}), staphylococcal nuclease (Ca^{2+}), cytochrome oxidase (Cu^{2+}/Cu^+ and Fe^{4+}/Fe^{3+}), and xylose isomerase (Mg^{2+} or Mn^{2+}) (Coleman & Gettins, 1983; Fersht, 1985; Stryer, 1988; Farber et al., 1989). Metal ions also contribute to RNA catalysis. Group I and group II introns have an absolute requirement for either Mg^{2+} or Mn^{2+}, as does ribonuclease P [reviewed by Cech and Bass (1986)]. Another group of RNA enzymes derived from plant viral satellite RNAs are greatly stimulated by a variety of divalent and polyvalent cations (Prody et al., 1986; Uhlenbeck, 1987).

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