Identification and Characterization of Cathepsin B as the Cellular MARCKS Cleaving Enzyme*

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The importance of regulating the cellular concentrations of the myristoylated alanine-rich C kinase substrate (MARCKS), a major cellular substrate of protein kinase C, is indicated by the fact that mice lacking MARCKS exhibit gross abnormalities of central nervous system development and die shortly after birth. We previously identified a novel means of regulating cellular MARCKS concentrations that involved a specific proteolytic cleavage of the protein and implicated a cysteine protease in this process (Spizz, G., and Blackshear, P. J. (1996) J. Biol. Chem. 271, 553–562). Here we show that p40, the carboxyl-terminal fragment resulting from this cleavage of MARCKS, was associated with the mitochondrial/lysosomal pellet fraction of human diploid fibroblasts and that its generation in cells was sensitive to treatment with NH4Cl. These data suggest the involvement of lysosomes in the generation and/or stability of p40. The MARCKS-cleaving enzyme (MCE) activity was associated with the mitochondrial/lysosomal pellet fraction of human diploid fibroblasts and that its generation in cells was sensitive to treatment with NH4Cl. These data suggest the involvement of lysosomes in the generation and/or stability of p40. The MARCKS-cleaving enzyme (MCE) activity was peripherally associated with a 10,000 × g pellet fraction from bovine liver, and it co-purified with the activity and immunoreactivity of a lysosomal protease, cathepsin B. Cathepsin B catalyzed the generation of p40 from MARCKS in a cell-free system and behaved similarly to the MCE with respect to mutants of MARCKS previously shown to be poor substrates for the MCE. Treatment of fibroblasts with a cell-permeable, specific inhibitor of cathepsin B, CA074-Me, resulted in parallel time- and concentration-dependent inhibition of cathepsin B and MCE activity. Incubation of a synthetic MARCKS phosphorylation site domain peptide with purified cathepsin B resulted in cleavage of the peptide at sites consistent with preferred cathepsin B substrate sites. These data provide evidence for the identity of the MCE as cathepsin B and suggest that this cleavage most likely takes place within lysosomes, perhaps as a result of specific lysosomal targeting sequences within the MARCKS primary sequence. The data also suggest a direct interaction between MARCKS and cathepsin B in cells and leave open the possibility that MARCKS may in some way regulate the protease for which it is a substrate.

The myristoylated alanine-rich C kinase substrate (MARCKS) is a prominent cellular substrate for protein kinase C (PKC) (1, 2). Expression of this heat-stable, acidic protein is essential for life, as demonstrated by the perinatal death of mice that are completely deficient in MARCKS (3). Complete lack of expression leads to gross abnormalities of central nervous system development; however, heterozygous mice, which express MARCKS at 50% wild-type levels, appear to be normal.

Cellular levels of MARCKS are regulated by both transcriptional and translational mechanisms (4–14). In addition, we recently demonstrated that the cellular concentrations of MARCKS can also be regulated by a proteolytic event. This proteolytic cleavage results in amino- and carboxyl-terminal fragments of MARCKS that co-exist in cells with the full-length protein. This cleavage of MARCKS was inhibited in intact fibroblasts by activation of PKC, concomitant with an increase in MARCKS levels. In a cell-free system, PKC-phosphorylated MARCKS was a poor substrate and unphosphorylated MARCKS was a good substrate for a cysteine protease that was capable of cleaving MARCKS into its two characteristic fragments (15). These data suggested that the phosphorylation site domain (PSD) of MARCKS might regulate the ability of MARCKS to serve as a proteolytic substrate. Amino-terminal sequence analysis of one of two carboxyl-terminal fragments purified from bovine spleen demonstrated that one site of cleavage was three amino acids amino-terminal to the PSD, implicating the PSD as a regulatory site for interaction with the protease rather than as the actual site of cleavage.

The purpose of the present study was to identify the intracellular cysteine protease responsible for this specific cleavage of MARCKS. Conventional purification methodology resulted in the partial co-purification from bovine liver of the lysosomal cysteine protease, cathepsin B, with the MARCKS-cleaving enzyme (MCE) activity. In addition, cathepsin B exhibited the same specificity toward MARCKS and its mutants as did the MCE. Lysosomal involvement in intact cells was confirmed by the sensitivity of the MCE activity to NH4Cl treatment. Using a cell-permeable, specific inhibitor of cathepsin B, CA074-Me, we demonstrated inhibition of the cellular MCE in human fibroblasts that was parallel in concentration dependence and time course with the inhibition of cathepsin B. Finally, using purified cathepsin B and synthetic substrates, we demonstrated that cleavage by cathepsin B occurs within the PSD of MARCKS. These data suggest that cathepsin B behaves as an MCE in cell-free systems and is the MCE in intact fibroblasts that is responsible for the PKC-regulated cleavage of MARCKS into two relatively stable products.
**MATERIALS AND METHODS**

**Cell Maintenance and Radiolabeling**—Human foreskin fibroblasts (HFF, Clonetics, San Diego, CA) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum, 1 mM penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). To serum starve cultures, confluent cultures were rinsed once with phosphate-buffered saline (PBS), and then medium was replaced with serum-free DMEM supplemented as above except that it contained 0.1% (v/v) bovine serum albumin (BSA), lyophilized and crystallized (Sigma). To label the cells with 1-[35]S]cysteine, DMEM lacking cysteine and methionine (Life Technologies, Inc.) was supplemented with 0.1% BSA, glutamine, penicillin, and streptomycin as above and 10 mM 1-mercaptoethanol. 1-[35]S]Cysteine (NEN Life Science Products) was added at 0.1–0.2 mCi/ml. For metabolic labeling, cells were serum-starved overnight in complete DMEM as above followed by transfer to labeling medium for the indicated times. For experiments involving NH4Cl, a 1:100 dilution of a 2 M stock solution was added for control treatment. Cells were incubated at 27 °C in a water-jacketed incubator supplemented with 5% CO2.

**Subcellular Fractionation**—Fibroblasts were grown on three 100-cm tissue culture dishes, serum-starved, and metabolically labeled as described above. Medium was aspirated, and cells were rinsed three times with ice-cold PBS. Using a rubber policeman, the cells were scraped into 1 ml of 10 mM Tris-HCl (pH 7.2) containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 200 nM leupeptin, and 574 μM phenylmethylsulfonyl fluoride. The dishes were rinsed with 2 ml of the same buffer for a final volume of 3 ml. The cells were homogenized with 30 strokes of a Wheaton glass homogenizer. Homogenates were centrifuged at 600 g for 10 min to remove nuclei and homogenized with 30 strokes of a Wheaton glass homogenizer. Homogenates were centrifuged at 10,000 g for 10 min. The resulting pellet was resuspended like supernatant, and the supernatant was collected at 0.5 ml/min using a 20-ml gradient of approximately 0–300 mM NaCl. After the fractions were collected on glass fiber filters, the filters were washed once with 2 ml of the same buffer and then frozen and stored in 25-ml aliquots at −70 °C.

For purification of the MARCKS-cleaving enzyme (MCE) activity—bovine liver was obtained fresh from the slaughterhouse, kept on ice during transit, and used within 1–2 h. The following procedures were performed at 4 °C. Approximately 200 g of tissue was rinsed and cut into smaller pieces in ice-cold PBS followed by homogenization in 7.5 volumes of buffer A: 10 mM Tris-HCl (pH 7.2) containing 250 mM NaCl, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 200 mM aprotinin, 1 mM benzamidine HCl, 1 μM pepstatin, and 574 μM phenylmethylsulfonyl fluoride. Leupeptin was eliminated from this buffer since we were assaying a leupeptin-sensitive protease. Homogenization was achieved with either a Waring type blender or Polytron mixer. Similar results were obtained using either method. For both methods, low speeds were used to avoid foaming. Homogenates were filtered through cheesecloth followed by centrifugation at 600 × g for 30 min. The resulting supernatants were centrifuged at 10,000 × g for 45 min. The resulting pellet was washed in buffer A, followed by two washes in buffer B, which is the same as buffer A except without NaCl and sucrose. The resulting pellet was resuspended in 400 ml of this buffer and then frozen and stored in 25-ml aliquots at −70 °C.

For purification, an aliquot was thawed, refrozen and thawed, and then centrifuged at 100,000 × g for 45 min. The pH of the supernatant was adjusted to 5.0 and was brought to approximately 6.9 with 1 N NH4OH. Ammonium sulfate was added slowly with stirring to a final saturation of 50%. This mixture was centrifuged at 12,000 × g for 30 min. Ammonium sulfate was added slowly with stirring to the resulting supernatant to bring the final saturation to 80%. This mixture was centrifuged at 12,000 × g for 30 min. The pellet was resuspended in 15 ml of buffer B. The NH4SO4 was removed through sequential concentration and dilution using a Centriprep-10 filtration unit following the manufacturer’s instructions (Amicon Inc.). A final solution was diluted to 10 ml with buffer B and applied to a 1-ml Mono-Q anion exchange column (Pharmacia Biotech Inc.). The column was developed using fast protein liquid chromatography. The sample was applied to the column at a rate of 0.5 ml/min in buffer B, followed by washing using the same conditions. When the absorbance at 280 nm reached base line, the salt gradient was initiated. Half-ml fractions were collected at 0.5 ml/min using a 20-ml gradient of approximately 0–300 mM NaCl. Protease inhibitors excluding leupeptin were included in all column runs.

When required, fractions eluted from the Mono-Q column were adjusted to 8% (v/v) glycerol and frozen at −70 °C. Enzyme activity remained stable upon thawing of these fractions. After the fractions containing the MCE activity were identified, they were pooled and prepared for separation by phenyl-Sepharose hydrophobic chromatography. Fractions were thawed and adjusted to 1.7 M NH4SO4. Using fast protein liquid chromatography, the sample was applied to a 1-ml phenyl-Sepharose column (Pharmacia Biotech Inc.) in buffer B adjusted to 1.7 M NH4SO4. Columns were washed in the same buffer, and column elution was initiated when the absorbance at 280 nm reached baseline. Column elution was carried out using a 30-ml gradient of 1.7 M to 0 M NH4SO4 in buffer B. Protease inhibitors excluding leupeptin were included in all chromatographic separations.

**Inhibitor Experiments**—The cell-permeable inhibitor, CA074-Me (Peptides International, Louisville, KY), was prepared as a 10 mM stock solution in dimethyl sulfoxide (Me2SO) according to manufacturer’s instructions. For concentration-response experiments, the 10 mM stock was diluted in Me2SO and then diluted in 5% FCS-containing media to different concentrations. These solutions were each diluted 1:100 in DMEM and then added to cell culture medium at a 1:100 dilution. Therefore, the final amount of Me2SO was 0.01% for all conditions, and 0.01% Me2SO was used as a control treatment.

For competition experiments, cells were serum-starved with or without label overnight as described above. The following day, appropriate dilutions of CA074-Me or Me2SO were added to medium, and cells were allowed to incubate for 4–5 h. Medium was removed, cells were rinsed three times with ice-cold PBS, and detergent extracts were prepared. Radiolabeled extracts were subjected to immunoprecipitation with a MARCKS-specific antibody and analysis by SDS-PAGE as described (15), and nonradiolabeled extracts were used for cathespin B and MCE assays (see below).

For pretreatment experiments, cells were serum-starved overnight in serum-free DMEM containing cysteine and methionine and supplemented with antibiotics, glucose, and 0.1% BSA (see above). The following day, cysteine/methionine-free DMEM supplemented with antibiotics, glucose, methionine, and 0.1% BSA was used to replace the serum-free medium and reconstituting medium overnight. To this was added a 1:100 dilution of 100 μM CA074-Me, prepared as above, in a final concentration of 1 μM, or an equivalent amount of Me2SO. Cells were pretreated with inhibitor or Me2SO for the indicated times. Following pretreatment, 1-[35]S]cysteine was added directly to the medium at 150 μCi/ml, and the cultures were allowed to incubate for another hour. The medium was removed, and cultures were prepared for analysis by immunoprecipitation of MARCKS as described above. For these exper-
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iments the PBS used to rinse the cells was supplemented with 2–4 μM leupeptin and 5 μM CA074-Me; cell lysis buffer was supplemented with 2–4 μM leupeptin and 5 μM CA074-Me in addition to standard protease inhibitors already present. This was done to make sure that the cells were continuously exposed to inhibitor following initial treatment.

MARCKS cDNA was subcloned into the pALTER-1 vector at unique XhoI and HindIII sites. The primer, 5'-CACAGGCGGAGGACCGGCCACGCGAGTTCGATAGGGGACC-GCAAAGAAAAAAAAGA-3', was used to place an initiator methionine within an optimum Kozak sequence directly upstream of Glu148. In addition, a HindIII site was placed upstream of the methionine so that dIII cut Bluescript KS dIII fragment representing p40 could be acid-precipitated material was subjected to two-dimensional gel electrophoresis using the gel system described (19). The proteins from the 10% SDS-PAGE gel were transferred to nitrocellulose and processed for immunoblot analysis as described below.

**Western Blot Analysis—**To assay for the presence of immunoreactive cathepsin B in purified fractions, aliquots were boiled in SDS sample buffer containing 11% SDS-PAGE and 4% glycerol. The fractions from phenyl-Sepharose chromatography, 500-μl fractions were translated and desalted and used in a Centricon-10 filter (Amicon). Separated proteins were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) in 192 mM glycine, 25 mM Tris (pH 7.6), and 20% (v/v) methanol using a Transphor apparatus ( Hoefer, San Francisco, CA). All steps in the following immunoblotting method were performed at room temperature. Nonspecific sites on filters were blocked by incubation and shaking for 1 h with 5% (w/v) instant milk in 20 mM Tris (pH 7.6) containing 137 mM NaCl and 0.3% (v/v) Tween 20 (TBST). Filters were briefly rinsed with TBST followed by incubation with a 1:5000 dilution in TBST of a rabbit polyclonal antibody generated against recombinant human cathepsin B (generously provided by Dr. John Mort, Joint Diseases Laboratory, Shriners’ Hospital for Crippled Children, Montreal, Quebec, Canada) (20, 21). Filters were then incubated with antibody for 1 h with shaking and then rinsed three times by shaking in TBST for 10 min each time. The filters were then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) at a 1:5000 dilution in TBST for 30 min with shaking and then rinsed three times in TBST for 10 min each time. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp.) following the manufacturer’s instructions.

For immunoblotting of MARCKS proteins, filters were incubated with a 1:100 dilution of a MARCKS-specific monoclonal antibody (15). When competing peptides were used, an equivalent volume of a 5 μM peptide solution and antibody were mixed and incubated for 1 h at 4 °C with tumbler prior to incubation with the filter.

**RESULTS**

**Presence of p40 within Fibroblasts Is pH-dependent—**As an initial step in identifying the protease responsible for the cleavage of MARCKS and the generation of p40, we attempted to determine whether this was a lysosomal event. Proteinolyis within lysosomes is dependent on an acidic environment (17, 22). Treatment of cells with ammonium chloride has been shown to increase lysosomal pH, resulting in the inhibition of normal lysosomal proteolytic events (22, 23). To determine if the generation and/or stability of p40 was pH-dependent, radiolabeled cells were treated with 20 mM NH₄Cl for different times; detergent extracts of these cells were then prepared and analyzed for the presence of immunoprecipitable p40 (Fig. 1). Within 5 min of treatment of cells with NH₄Cl, the fraction of p40 was decreased by 32%; this continued to decrease with increasing times of NH₄Cl treatment, resulting in its complete disappearance by 20 min. In contrast, immunoprecipitates from cells treated with H₂O retained levels of p40 similar to control. These data suggest that the presence of p40 within cells appears to be dependent on lysosomal pH.

**p40 Is Enriched in the 10,000 × g Fraction of Cell Homogenates—**Preliminary data demonstrated that an activity that was sensitive of cleavage of MARCKS and generating p40 was associated with a 10,000 × g pellet fraction of bovine and murine liver homogenates. To determine if p40 was associated with the fraction, human fibroblasts were labeled with [³⁵S]cysteine, and the peptides generated were fractionated by SDS-PAGE. As shown in Fig. 2, full-length MARCKS was present in both the 10,000 × g (lane 1) and 100,000 × g pellets (lane 3), with a small fraction in the 100,000 × g cytosol (lane 5). P40 was detected in both the 10,000 × g and 100,000 × g pellets (lanes 1 and 3) but was absent in the cytosolic fraction (lane 5). Densitometry demonstrated that p40 was present at approximately 33 and 13% total immunoreactive MARCKS in the 10,000 × g pellet and the 100,000 × g pellet, respectively. This experiment was repeated four times with similar results.
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Fig. 1. The amount of p40 in fibroblasts is sensitive to NH4Cl treatment. A, human fibroblasts labeled overnight with [35S]cysteine were treated with H2O (−) or 20 mM NH4Cl (+) for the indicated times. Detergent extracts of the cells were subjected to immunoprecipitation and analyzed as described under “Materials and Methods.” The arrows indicate MARCKS (top) and p40 (bottom). B, results of densitometric scans of the autoradiogram in A. The amount of p40 was calculated as the fraction of total immunoprecipitable MARCKS, and each point represents the percentage of the values from the water-treated cells at time 0. Solid bars, control; hatched bars, NH4Cl.

These data demonstrated a relative enrichment of p40 in the 10,000 × g pellet fraction of human fibroblasts, compared with cytosolic and 100,000 × g pellet fractions.

We also tested the subcellular distribution of MARCKS and p40 in cells treated with NH4Cl (Fig. 2). Radiolabeled cells were treated with NH4Cl or control conditions for 15 min, and homogenates were fractionated into membrane and cytosol fractions which were then subjected to immunoprecipitation with a MARCKS-specific antibody. Following treatment with NH4Cl, the fraction of p40 in the 10,000 × g pellet fraction decreased from 33 to 4% (compare lanes 1 and 2), and it was no longer detectable in the 100,000 × g pellet fraction (compare lanes 3 and 4). In contrast, MARCKS was still readily detectable in both the 10,000 × g and 100,000 × g pellet fractions (lanes 2 and 4). These data demonstrate that either or both the stability and the generation of p40 appear to be sensitive to NH4Cl treatment but not the mechanism by which MARCKS gets targeted to the appropriate cellular location.

The disappearance of p40 in response to NH4Cl treatment could be due to further proteolysis of p40, its secretion, or inhibition of the proteolytic event responsible for its generation from full-length MARCKS. Taken together, the enrichment of p40 in the 10,000 × g pellet fraction and its sensitivity to NH4Cl are consistent with the possibility that the generation of p40 is a lysosomal event.

Partial Purification of the MARCKS-cleaving Enzyme—The above data suggested that the activity responsible for cleaving MARCKS and generating p40 might be a lysosomal protease. Lysosomes contain many cathepsins that are responsible for the degradation of proteins targeted to these vesicles (24). We previously demonstrated that the protease activity responsible for generating p40 was a cysteine protease (15). Cysteine proteases present within the lysosome include cathepsins B, L, H, and S (24) as well as the more recently described O2 and O (25–31). We considered the possibilities that the MCE could be one of these known proteases or a novel protease. We were able to exclude most of the previously described lysosomal cysteine proteases as the MCE based on tissue expression or biochemical properties (see “Discussion”), except for cathepsin B. We therefore attempted to purify the MCE activity using the assay described previously (15); we also assayed cathepsin B activity (17) and immunoreactivity in the same fractions.

Using fresh bovine liver as a starting material, we determined that the MCE activity was preferentially associated with the 10,000 × g membrane pellet fraction of cell homogenates. The activity did not require detergent to be released from this membrane fraction but could be released by two cycles of freezing and thawing (data not shown). These observations suggested that the activity was not an integral membrane protein and were consistent with it being a lysosomal protease. The activity was enriched in a 50–80% NH4SO4 fraction (data not shown). This material was then subjected to Mono-Q anion exchange column chromatography (Fig. 3A). The peak of enzyme activity eluted at approximately 117 mM NaCl, immediately after the major peak of protein. The peak of cathepsin B activity was identical to that of the MCE activity (Fig. 3A).

Fractions enriched with MCE activity were then subjected to hydrophobic column chromatography (Fig. 3B). The peak of MCE activity eluted at approximately 800 mM NH4SO4, immediately following a major protein peak. Once again the peak of cathepsin B activity was identical to that of the MCE activity.

The exact co-purification of both the MCE activity and cathepsin B activity through two separate chromatographic steps suggested that cathepsin B was a good candidate for the MCE.

Although the substrate used for the cathepsin B assays is known not to be recognized by other cathepsins of the cysteine protease class (17), we also assayed the column fractions by immunoblotting with a polyclonal antiserum to recombinant human cathepsin B. Cathepsin B purified from bovine spleen was used as a positive control. The anti-cathepsin B antibodies recognized the peak fractions of cathepsin B/MCE activity as well as the control cathepsin B (Fig. 3, A and B, insets). The antibody detected three major protein bands in both the control sample and the Mono-Q peak fractions of enzyme activity, of approximate Mr values of 32,000, 27,000, and 25,000 (Fig. 3A, inset). These sizes are consistent with those described in a previous report of cathepsin B purification from bovine liver (32). The 32-kDa form most likely corresponds to a single chain un-
cleaved form, and the 25–27-kDa form corresponds to the heavy chain of the double-stranded form of the enzyme. The doublet at 23–25 kDa is most likely due to differences in carbohydrate chain of a double-stranded form of the enzyme. The doublet at 23–25 kDa is most likely due to differences in carbohydrate chain of a double-stranded form of the enzyme. The doublet at 23–25 kDa is most likely due to differences in carbohydrate chain of a double-stranded form of the enzyme. The doublet at 23–25 kDa is most likely due to differences in carbohydrate chain of a double-stranded form of the enzyme. The doublet at 23–25 kDa is most likely due to differences in carbohydrate chain of a double-stranded form of the enzyme.

**Fig. 3.** Chromatographic elution profiles and immunoblot analysis of the MCE and cathepsin B activities. Extracts of fresh bovine liver membranes were subjected to Mono-Q anion exchange chromatography (A) followed by phenyl-Sepharose hydrophobic chromatography (B). Column fractions were assayed for cathepsin B and MCE activities. Fractions including the peak fractions of cathepsin B and MCE activity were also analyzed for the presence of immunoreactive cathepsin B (insets). —, salt gradient; M, A528; A, cathepsin B activity; M, MCE activity; Bovine, purified bovine cathepsin B; Sm, starting material originally applied to the column. Numbers in both insets correspond to the fraction numbers from the respective column elution profiles. Arrows indicate the 32-kDa single chain, uncleaved form of cathepsin B (upper arrow in A) and the 25–27-kDa heavy chain of the double-stranded form of the enzyme (lower arrow in A and arrow in B).

We next tested the ability of purified cathepsin B to demonstrate that the MCE activity co-purifies with cathepsin B activity and immunoreactivity and support the identity of cathepsin B as the MCE. In addition, we are unaware of a previous example of a substrate for cathepsin B whose cleavage is regulated by phosphorylation/dephosphorylation.

**CA074-Me, a Specific Cell-permeable Inhibitor of Cathepsin B, Inhibits the MCE Activity and the Generation of p40 in Human Fibroblasts**—We next determined whether cathepsin B is the enzyme responsible for cleaving MARCKS to p40 in intact cells. The synthetic inhibitor CA074 and its cell-permeable methyl ester derivative, CA074-Me, have been shown to be specific for cathepsin B (34–36). We therefore studied the ability of the cell-permeable inhibitor to inhibit the generation of p40 in human fibroblasts.

Serum-starved HFF were next treated with CA074-Me for 4–5 h and then assayed for immunoreactive p40, cathepsin B activity, and MCE activity. As shown in Fig. 5A, all three assays were affected in parallel by different concentrations of the inhibitor. Both enzyme assays exhibited 50% inhibition between 10 and 100 nM inhibitor; the generation of p40 in intact cells was inhibited by approximately 25% by these concentrations. However, 1 μM inhibitor abolished all three activities (Fig. 5A). This concentration of inhibitor is similar to that used to inhibit cathepsin B in human gingival fibroblasts (36).

We also tested concentrations of inhibitor between 10 nM and 1 μM for the inhibition of cathepsin B and MCE activity. Both activities were inhibited in parallel, demonstrating a linear inhibition between 10 and 100 nM, followed by an apparent plateau at 500 nM (Fig. 5), further supporting the identity of cathepsin B as the MCE.

HFF were next treated with inhibitor (1 μM) for times up to 120 min and then assayed for cathepsin B activity and the generation of p40. Treatment of the fibroblasts with inhibitor for 5 min reduced cathepsin B activity in cellular homogenates.
Cathepsin B Cleaves MARCKS within the PSD—To confirm that cathepsin B cleavage of MARCKS occurred at the previously described cleavage site (15), we constructed a cDNA clone coding for a protein equivalent to the p40 fragment of MARCKS that would result from cleavage three amino acids upstream of the PSD. In vitro translation of this fragment followed by SDS-PAGE demonstrated that it migrated slightly more slowly than p40, generated either from cathepsin B cleavage of MARCKS or from HFF (Fig. 7, compare lane 2 with lanes 3 and 5). We therefore asked whether the “p40” generated from this cDNA construct was a substrate for cathepsin B. Fig. 7 demonstrates that cleavage of this in vitro translated p40 by cathepsin B resulted in a faster migrating band that comigrated both with the p40 from HFF and that produced by cleavage of MARCKS with cathepsin B (Fig. 7, compare lane 4 with lanes 3 and 5). This cleavage was most likely within the amino-terminal portion of p40, since the epitope recognized by this antibody is near the carboxyl terminus of MARCKS.2 This suggested that in addition to the cleavage site determined by amino-terminal sequencing of one of two bands representing bovine spleen p40 (15), there might be one or more additional sites recognized by MARCKS within MARCKS recognized by cathepsin B. The slightly faster migration of p40 generated from in vitro translated MARCKS is presumably due to its bovine origin, whereas all the other lanes correspond to p40 fragments of human MARCKS; the calculated Mr for the carboxyl-terminal fragment of bovine MARCKS is slightly lower than that for human MARCKS (2).

Because phosphorylation of MARCKS inhibited the generation of p40, and the p40 seen in HFF was slightly (approximately 3 kDa) smaller than that predicted from the bovine spleen fragment, it seemed possible that cathepsin B might cleave MARCKS within the PSD. To test this possibility, we subjected a purified 25-amino acid synthetic PSD peptide to cleavage with cathepsin B. Incubation of the PSD peptide with cathepsin B decreased its apparent Mr, on SDS-PAGE by approximately 50% (data not shown). This cleavage of the PSD peptide by an excess of cathepsin B appeared to be complete.

2 P. J. Blackshear, unpublished observations.
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FIG. 6. Time course of inhibition by CA074-Me of cathepsin B and MCE activities in human fibroblasts. Fibroblasts were serum-starved overnight followed by treatment with MeSO or 1 μM CA074-Me for the indicated times. A, detergent extracts were assayed for cathepsin B activity as described under “Materials and Methods.” Results represent cathepsin B activity/ng of protein and are expressed as a percentage of the untreated controls. B, following treatment with MeSO or 1 μM CA074-Me for the indicated times, fibroblasts were labeled with [35S]cysteine for 1 h in the continued presence of inhibitor. Detergent extracts were prepared and assayed for the presence of p40 by immunoprecipitation, as described in the legend to Fig. 1. The graph represents the results of scanning densitometry of the autoradiogram. The amount of p40 is expressed as the percentage of total immunoprecipitable MARCKS from cells treated with MeSO at time 0. A and B, solid bars, MeSO; hatched bars, CA074-Me. Each point represents the mean ± S.D. of three values.

FIG. 7. Cathepsin B cleaves in vitro translated p40. In vitro translated, non-myristoylated bovine MARCKS (lanes 1 and 3) and p40 (lanes 2 and 4) were incubated at 37 °C for 30 min with (lanes 3 and 4) or without (lanes 1 and 2) 0.09 units of cathepsin B. Lane 5, boiled and clarified detergent extract of human fibroblasts labeled with [35S]cysteine overnight. All samples were subjected to immunoprecipitation as described under “Materials and Methods” and in the legend to Fig. 1. The positions of protein molecular weight standards are indicated.

by 30 min, with no further digestion detected up to 2 h of incubation.

We then subjected the peptide products generated by cathepsin B cleavage of the PSD peptide to reverse-phase HPLC and demonstrated the presence of two major peaks (data not shown); these were subjected to amino-terminal sequencing. Cleavage by cathepsin B occurred at least at two sites within the PSD (Fig. 8), one of which is consistent with a consensus cathepsin B cleavage site (see “Discussion” and Fig. 8). This HPLC analysis was repeated with similar results; in neither instance was the amino-terminal portion of the PSD recovered. One possible explanation is that the presence of five lysines and an arginine at the amino terminus may result in a fragment of the PSD that is too hydrophilic to bind to the column under the conditions employed here. Alternatively, cathepsin B might cleave within the amino portion of the peptide at one or more additional sites in addition to the known sites (see Fig. 8).

The amino-terminal sequence described in our previous paper (15) represented the predominant upper band of a doublet of p40 from bovine spleen. We subjected a similar preparation to two-dimensional SDS-PAGE followed by immunoblotting with a carboxyl-terminal specific MARCKS antibody (Fig. 9) in the absence (upper panel) or presence (lower panel) of an excess of competing epitope peptide. MARCKS itself appeared as a series of spots migrating at approximately Mr 80,000; the multiple spots are presumably the result of different phosphorylated species (37). In addition, there were several immunoreactive spots migrating at approximately Mr 40,000, with minor differences in pI and in Mr. The most prominent spot migrates at the greatest apparent Mr and most basic pI, consistent with it being the p40 species that was sequenced in our earlier paper (15). There was a second series of spots of lower Mr and pI, consistent with the loss of 6–8 basic residues from the amino terminus of the PSD. These data provide additional evidence for the idea that, although the upper band of the p40 doublet present in bovine tissues results from cleavage three amino acids upstream of the PSD, the lower band represents MARCKS cleaved at sites within the PSD; one or more of these PSD sites is likely to be the site(s) involved in p40 generation in intact HFF.

DISCUSSION

We previously described a proteolytic activity in human fibroblasts and mouse and bovine tissues that was responsible for the cleavage of MARCKS, resulting in the production of two stable fragments of Mr 44,000 and 40,000 (p40) that co-exist in cells with the full-length protein (15). Based on inhibitor studies, the protease responsible for this cleavage appeared to be of the cysteine class. The aim of the present study was to identify this cellular protease.

Initial data on subcellular localization and pH sensitivity of the MCE activity suggested that it was a lysosomal cathepsin. Several lysosomal cathepsins of the cysteine protease class demonstrate endoproteolytic activity. These include the well studied cathepsins B, L, S, and H and the more recently described O and O2 (also referred to as K, O, and OC2) (17, 18, 37, 44, 45). Cathepsin B (a serine protease) does not fit this profile, whereas the MCE activity specific for MARCKS cleavage is inhibited by CA074-Me (15). This agent is a specific, reversible inhibitor of a family of aspartyl proteases (35) that include the cathepsins L, S, and H. By contrast, it is a highly specific, irreversible inhibitor of the cysteine cathepsins: B, L, and H.

The results reported here strongly suggest that the cellular protease responsible for the cleavage of MARCKS is a cysteine protease, probably a cathepsin. For example, we show that the activity is inhibited by CA074-Me and that it is a lysosomal enzyme (15). Cathepsin B cleaves the PSD from the full length MARCKS within the amino terminus of the PSD, resulting in the production of two stable fragments of Mr 80,000 and 40,000 (37). Such a cleavage results in the production of a stable fragment that is characteristic of the MCE activity described here.
standards are indicated. The additional spots were visualized by enhanced chemiluminescence. The pH gradient was achieved using a carrier ampholyte (pH 4–6) and the gel was run at 200 mA for 16 hours. The gel was then stained with Coomassie Blue. The molecular mass markers were: 97, 66, 45, 29, 11 kDa. The autoradiograph shows the detection of MARCKS at a molecular mass of approximately 40 kDa.

We have also demonstrated that the MCE is inhibited by the specific inhibitor CA074-Me, which causes a 50% inhibition of cellular cathepsin B activity within 10 min and essentially complete inhibition within 60 min, when assayed in extracts of treated cells. Similarly, cellular levels of p40 were decreased by approximately 40% following inhibitor treatment for 10 min and continued to decrease until 60 min. Taken together, these data provide strong evidence that cathepsin B is the MCE that generates p40 in intact cells.

The association of p40 with the lysosomal fraction of cells and the probable identity of the MARCKS-cleaving enzyme as the lysosomal protease cathepsin B suggest that the cleavage of MARCKS is a lysosomal event. This leads to the question of how MARCKS gets targeted to the lysosome. MARCKS is associated with the cellular plasma membrane predominantly by two mechanisms, the hydrophobic association of the amino-terminal myristate group with lipids of the membrane bilayer (16, 44, 45), and the electrostatic interaction of the highly basic phosphorylation site domain with acidic phospholipids of the membrane (16, 45–49). Following PKC-mediated phosphorylation of MARCKS, its affinity for the membrane is decreased and cytosolic MARCKS is increased (16, 45, 50–52). Therefore, although peripherally associated with the plasma membrane, MARCKS is also present in the cytosol, and it is not obvious how it becomes exposed to proteases present within lysosomes.

There are several mechanisms by which cytosolic proteins get targeted to the lysosomes for proteolytic destruction. One mechanism is a non-selective, bulk, vesicular process of uptake of cytosolic molecules, including proteins and RNA, known as autophagy (53, 54). This mechanism is regulated by small GTPases (55, 56, 57) and with the lysosomal marker glycoprotein, Lgp96 (LAMP2), recently demonstrated to behave as a receptor for the targeted cytosolic proteins (58).

In addition, selective uptake is also dependent on a specific pentapeptide motif originally identified in a 20-amino acid amino-terminal peptide of bovine ribonuclease A (59–61). This sequence is (K,R)(F,I,L,V)(E,D)(X) flanked on either side by a glutamine residue. X denotes any amino acid but is usually a highly hydrophobic, basic, or acidic residue. In addition, although the glutamine residue must be flanking, the order of the remaining four residues is unimportant (60). The sequence KEE(L,V)Q, consistent with this motif, is present in MARCKS proteins from all species known to date (2). This motif resides in the amino half of the protein, 16 amino acids carboxy-terminal of the mannose 6-phosphate/insulin-like growth factor II receptor homology region. Further experiments will be necessary to determine if MARCKS is susceptible to selective lysosomal uptake through this motif. For example, mutagenesis of the postulated selective uptake motif will provide evidence of whether or not this mechanism is involved in the lysosomal targeting of MARCKS.

Specific targeting of MARCKS to the lysosome is consistent
with a previous report by Aderem and colleagues (62), which discussed the association of phosphorylated, but not non-phosphorylated, MARCKS with the lysosome. We have demonstrated that the phosphorylation state of MARCKS regulates its proteolysis. If selective uptake is shown to be the mechanism by which MARCKS gets targeted to the lysosome, it will also be of interest to determine whether phosphorylation of MARCKS also affects this process.

The specificity of the inhibitor, CA074 (N-[(3-propylcarbamoyl)-2-carbonyl]-i-isoleucyl-i-proline), and that of its cell permeable form, CA074-Me, for cathepsin B have been established by both cell-free and intact cell studies (34–36) and by modeling based on x-ray crystallographic data (36, 63). In one study, CA074 exhibited rates of inactivation of cathepsin B that were more than 1000-fold greater than those seen with cathepsins H, L, and S (36). Similarly, Murata et al. (34) demonstrated that the IC50 of CA074 for cathepsin B was 1.3 × 10−5 and 5.3 × 10−6 of those for cathepsins L and H, respectively. In another study, rats were injected intraperitoneally with CA074, and cathepsin activities were assayed in a crude mitochondrial/lysosomal fraction of liver (35). Cathepsin B activity was essentially undetectable in these preparations, whereas the activities of cathepsins L and H remained similar to those found in control animals.

Since CA074 is a highly negatively charged molecule, the cell-permeable methyl ester derivative, CA074-Me, has been used for experiments in intact cells (36). CA074-Me is an ineffective proinhibitor of cathepsin B in cell-free systems, due to the esterification of the carbonyl group of the carboxyl-terminal proline (36). However, upon entering cells, CA074-Me is thought to be de-esterified to CA074, the specific inhibitor of cathepsin B (36).

In our original description of MARCKS cleavage (15), we demonstrated the presence of a carboxyl-terminal fragment of MARCKS in bovine tissues that migrated similarly to p40 in intact HFF. We show here that if the same material from bovine spleen is subjected to two-dimensional SDS-PAGE followed by immunoblotting, several species of immunoreactive peptides are apparent of approximately Mr 40,000. These data are consistent with the presence of several related fragments of MARCKS of nearly the same size. Based on sequence data from the largest spot, we showed that one cleavage site was present three amino acids amino-terminal to the PSD (15). In the present report, we demonstrate that cathepsin B cleaves MARCKS at least two sites within the PSD. The slower migrating band identified in spleen has never been detected in HFF nor do we detect it upon subjecting MARCKS to cathepsin B digestion in cell-free systems. In addition, a cDNA representing this slower migrating band encodes a protein fragment that is a good substrate for cathepsin B, resulting in a fragment of essentially identical Mr to that seen in HFF. At this point it is not clear if the larger p40 is a product of cathepsin B cleavage or of a different protease. However, based on the data in this report, cathepsin B cleaves MARCKS and generates p40 in HFF, and one or more of these cleavages is in the PSD.

Several studies have investigated the substrate specificities of the endo- and exo-peptidase activities of cathepsin B (21, 64–66). Although one such study used soluble, denatured proteins as substrates (65), most studies have employed synthetic peptides to analyze the interactions between the active site of the enzyme with the substrate cleavage site (21, 64, 66). From these studies it has been suggested that the endopeptidase activity of cathepsin B prefers, but is not limited to, a basic and hydrophobic amino acid at the P1 and P2 substrate sites, respectively (65). As we have shown in this paper, cathepsin B recognizes at least two sites within the PSD of MARCKS, one of which is consistent with sites preferred by the protease (Peptide A, Fig. 8). The sequence we originally reported for the MARCKS cleavage (15) contains an asparagine in the P1 position of human, bovine, and Xenopus MARCKS,4 or a serine in chicken, mouse, and rat MARCKS; all the known MARCKS sequences contain a serine at the P2 position (2). Although these residues do not appear to be optimum for a cathepsin B site, a serine at P1 and P2 was seen in two of the sequences analyzed for cleavage by cathepsin B by Koga et al. (65), and it is therefore possible that cathepsin B may be responsible for the generation of the larger p40.

We have shown that in the unphosphorylated state, the highly basic PSD of MARCKS allows cleavage by cathepsin B; however, when the serines within the PSD become phosphorylated or are replaced by aspartates, MARCKS is no longer a good substrate for cathepsin B (Fig. 4A and Ref. 15). It is unknown at this point which phosphorylated serine is responsible for the inhibition of MARCKS cleavage by cathepsin B. It is known that the first, second, and fourth serines, but not the third serine, within the PSD are the targets of PKC-mediated phosphorylation (67, 68). Although there are three potential optimal cathepsin B sites within the PSD, we have detected cleavage at only one of these preferred sites in vitro (Peptide A, Fig. 8). It has been reported that Glu423 in human and rat cathepsin B plays a significant role in the interaction of the enzyme with its substrates (64, 66). It is tempting to speculate that PKC-mediated phosphorylation of the second serine within the PSD is responsible for inhibiting the cleavage at the site represented by Peptide A (Fig. 8) and that this inhibition is the result of new acidic charges in the PSD interfering with the glutamate interaction necessary for binding, thereby preventing interaction of the protease with MARCKS.

To our knowledge, there are no previous examples of cathepsin B cleavage of a physiological substrate that is inhibited by phosphorylation of the substrate. However, examples of such substrate modification have been described for other proteases. For example, PKC-mediated phosphorylation of connexin-32 (69), and protein kinase A-mediated phosphorylation of the microtubule-associated proteins MAP-2 and tau (70, 71), protected them from calpain-mediated proteolysis.

We previously reported that whereas the MCE activity was detected in crude homogenates of spleen, liver, and kidney, it was not detected in similar homogenates from brain (15). However, further analysis demonstrated the same MCE activity in an extracted membrane fraction from brain homogenates.3 The inability to detect this activity in crude homogenates, but its ready detection in membrane extracts, is consistent with the fact that high concentration of soluble cysteine protease inhibitors, cystatins, are known to be present in brain (72–74).

At this point the physiological significance of constitutive MARCKS cleavage by cathepsin B in fibroblasts and other cells and tissues is uncertain. However, it also seems possible that MARCKS may regulate in some way the action or secretion of cathepsin B. For example, it is known that cathepsin B can degrade laminin, fibronectin, and collagen (75–77) and can activate the urokinase-type plasminogen activator associated with tumor invasiveness (78). Studies from our laboratory have shown that the brains of mice completely lacking MARCKS exhibit deficiencies in at least two types of extracellular matrix molecules, laminin and chondroitin sulfate proteoglycans (79). It is conceivable that these matrix abnormalities might involve excessive action or secretion of cathepsin B in the MARCKS-deficient animals.

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