Mitochondrial calpain 10 activity and expression in the kidney of multiple species

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Abstract

Calpains, Ca^{2+}-activated cysteine proteases, have been implicated in the progression of multiple disease states. We recently identified calpain 10 as a mitochondrial calpain that is involved in Ca^{2+}-induced mitochondrial dysfunction. The goals of this study were to characterize the expression and activity of renal mitochondrial calpain 10 in rabbit, mouse, and rat. Using shRNA technology and immunoblot analysis three previously postulated splice variants of calpain 10 were identified (50, 56, and 75 kDa). SLLVY-AMC zymography and immunoblot analysis was used to directly link calpeptin-sensitive calpain activity to calpain 10 splice variants. Rabbit, mouse, and rat kidney mitochondria contained 75 kDa (calpain 10a), 56 kDa (calpain 10c or 10d), and 50 kDa (calpain 10e). Interestingly, zymography yielded distinct bands of calpain activity containing multiple calpain 10 splice variants in all species. These results provide evidence that several previously postulated splice variants of calpain 10 are localized to the mitochondria in kidneys of rabbits, rats and mice.

Keywords

calpain 10; mitochondria; kidney; SLLVY-AMC; zymography

INTRODUCTION

Calpains are a ubiquitously expressed 15-member family of Ca^{2+}-activated cysteine proteases that have been implicated in many disease states (e.g. muscular dystrophy, gastric cancer, type II diabetes, and renal failure) [1;2;3;4;5;6]. The calpain family is divided into two groups, typical and atypical. The first group (calpains 1, 2,3,8,9,11,12,14), are known as typical calpains because they are comprised of four domains including the Ca^{2+} binding domain (domain IV). The second group (calpains 5,6,7,10,13,15) are known as atypical calpains because they lack the Ca^{2+} binding domain (domain IV) [1].

Calpain 10 is an atypical calpain that has recently gained attention due to its potential involvement in type 2 diabetes. In 2000 a genome wide scan for type II diabetes susceptibility genes in a population of Mexican Americans identified the calpain 10 gene (CAPN10) as a putative type 2 diabetes susceptibility gene [4]. Since then, multiple other studies involving diverse populations have supported this finding, while others have not [7]. Other investigators

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have linked this genetic association of CAPN10 and type 2 diabetes to functional roles for calpain 10 in the progression of the diabetic phenotype, including regulation of glucose uptake via GLUT4 vesicles [8;9] and regulation of mitochondrial metabolism and insulin secretion [10].

While calpains are generally thought to be cytosolic, our laboratory recently identified calpain 10 as a mitochondrial calpain and demonstrated that it plays a role in Ca^{2+}-induced mitochondrial dysfunction [11]. Specifically, rabbit mitochondrial calpain 10 has a mitochondrial targeting sequence and is responsible for Ca^{+2}-induced cleavage of Complex I proteins NDUFV2 and ND6. Horikawa et al [4] described the genetics of human CAPN10, specifically the ability of the gene to undergo alternate splicing, yielding eight potential gene products of varying size. To date there has not been conclusive evidence that the protein products of CAPN10 splice variants are expressed, although multiple investigators have identified immunoreactive bands that correspond to the predicted molecular weight of the splice variants [12;13;14]. Thus, the aims of this study were to determine whether multiple calpain 10 splice variants exist in mitochondria and to determine the expression and activity of calpain 10 across species.

MATERIALS AND METHODS

Reagents

Calpain 10 antibody and HRP-conjugated goat anti-rabbit secondary antibody were purchased from Abcam (Cambridge, MA) and Pierce (Rockford, IL), respectively. SLLVY-AMC and Percoll were obtained from Bachem (King of Prussia, PA) and Amersham Biosciences (Piscataway, NJ), respectively. Calpeptin and purified porcine calpain 1 were purchased from Calbiochem (La Jolla, CA). Dulbecco's Modified Eagle Medium, calf serum, and lipofectamine were obtained from Invitrogen (Carlsbad, CA) and shRNA plasmids targeted to calpain 10 were purchased from Origene (Rockville, MD). All other chemicals were obtained from Sigma (St. Louis, MO).

Calpain 10 shRNA

Normal rat kidney (NRK-52E) cells were cultured as previously decribed [15]. Calpain 10 shRNA was transfected into NRK-52E cells using lipofectamine. After 48 hr cells were lysed and immunoblot analysis was preformed.

Mitochondrial isolation

Mitochondria were isolated from the kidney cortex of female New Zealand White rabbits (2 kg), kidney cortex of male Sprague-Dawley rats (250 g) and whole kidney of male C57BL/6 mice (20–30 g) as previously described [11;16]. Following isolation of kidney mitochondria from the rabbit, further fractionation was performed to yield a mitochondrial matrix fraction as previously described [11;17].

Cellular Fractionation

Renal proximal tubules were isolated from New Zealand White rabbits (2 kg) using the iron oxide perfusion method and grown in 35-mm culture dishes as previously described [18;19]. Cells were grown to confluence and harvested for isolation of mitochondrial and cytosolic fractions as previously described [20].

Zymography

Zymography was preformed as previously described with modifications [11;21]. Briefly, zymogram gels were cast just prior to use and were composed of 8% acrylamide for resolving

and 6% acrylamide for stacking. Resolving gels were copolymerized with the calpain substrate SLLVY-AMC (5 μM). Mitochondrial protein samples, briefly sonicated prior to electrophoresis to release proteins (50–100 μg), and purified porcine calpain 1 (10 μg) were electrophoretically separated under nondenaturing conditions (running buffer – 25 mM Tris base, 125 mM glycine, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.0) at 125 V for 3 hours at 4°C. Following electrophoresis, gels were incubated in a Ca^{2+} buffer (50 mM Tris-HCl, 5mM $CaCl_2$, 10 mM 2-mercaptoethanol, pH 7.0) for 10 min at room temperature and imaged on an Alpha Innotech imaging station. The presence of fluorescent bands indicated proteolytic activity. Bands of activity were excised from the zymogram and proteins were eluted from the gel slices in a denaturing elution buffer (25 mM Tris-base, 192 mM glycine, 1 mM thioglycolic acid, and 0.1% SDS, pH 8.8). Eluted proteins were concentrated in protein concentrating microcentrifuge tubes (Millipore) and subjected to immunoblot analysis.

Immunoblot analysis

Protein samples were separated by SDS-PAGE (4–12% acrylamide) and transferred to nitrocellulose membranes. Membranes were blocked in 2% non-fat milk in TBST-20 (Tris-buffered saline Tween-20), incubated with an antibody to Domain III of calpain 10 (1:1000) for 2 h, and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000) for 1 h. Immunoreactive proteins were visualized by enhanced chemiluminescence detection on an Alpha Innotech imaging system.

Calpain Activity

Calpain activity was measured spectrofluorometrically using the calpain substrate SLLVY-AMC as previously described [11;22]. Mitochondria or mitochondrial matrix fractions were incubated with SLLVY-AMC (50 μM) in the absence and presence of the calpain inhibitor, calpeptin (10 μM), in a buffer (130 mM KCl, 9mM Tris-PO_4, 4 mM Tris-HCl, and 1 mM EGTA, pH 7.4). Activity was measured under linear conditions as a function of SLLVY-AMC hydrolysis using excitation and emission wavelengths of 355 nm and 444 nm respectively.

Statistical analysis

Mitochondria or matrix isolated from one animal represents one experiment (n=1). Data are presented as means ± SE and were subjected to student's t-test. A value of $p<0.05$ was considered a statistically different.

RESULTS AND DISCUSSION

NRK-52E cells were transfected with a shRNA construct targeted to calpain 10 or scramble control, and immunoblot analysis was performed on cell lysates 48 h later. Using a calpain 10 specific antibody, three postulated splice variants were identified; 75 kDa, 56 kDa, and 50 kDa (fig 1a). The molecular weight of these postulated splice variants are consistent with molecular weight predictions of the gene products of the splice variants calpain 10a (75 kDa, full-length), calpain 10c or calpain 10d (56 kDa), and calpain 10e (50 kDa). In cells transfected with calpain 10 targeted shRNA, there was a decrease in protein expression of the 75 kDa (81%), 56 kDa (71%), and 50 kDa (60%) (fig 1a). There was no change in the levels of these proteins in cells transfected with the scramble control. The differences in the relative "knockdown" of the calpain 10 splice variants could be due to different protein stability and turnover or the efficiency of the shRNA to bind to and interfere with the mRNA of the splice variants. Although different antibodies and constructs were used, similar knockdown of the 75 kDa and 56 kDa splice variants was seen in primary cultures of human skeletal muscle cells that were transfected with siRNA targeted to calpain 10 [14]. These results provide strong evidence that the three immunoreactive bands recognized by the calpain 10 specific antibody are indeed three splice variants of calpain 10.

To examine the mitochondrial calpain activity in different species, the fluorogenic calpain substrate SLLVY-AMC was utilized. Because SLLVY-AMC is not specific for calpains [23; 24;25;26], we used the calpain inhibitor calpeptin to confirm calpain activity. Isolated mitochondria or matrix from rabbit, mouse, and rat were assayed for SLLVY-AMC cleavage over time in the presence or absence of 10 μM calpeptin (fig 1b). Calpeptin inhibited 86±5% (rabbit), 70±1% (mouse) and 85±7% (rat) of total SLLVY-AMC cleavage, indicating that substrate cleavage is primarily calpain mediated.

To further examine rabbit renal mitochondrial calpain activity, mitochondrial matrix proteins were separated under native conditions by SLLVY-AMC zymography. Following electrophoresis, gels were bathed in a Ca^{2+} buffer and imaged. Purified cytosolic calpain 1 was used as a control to visualize the migration pattern of this typical calpain. As previously reported matrix fractions yielded three major bands of activity that did not co-migrate with purified cytosolic calpain 1 (fig 2, A,B,C), indicating that the mitochondrial matrix calpain is not calpain 1 in rabbit renal cortical mitochondrial matrix [11]. Bands of activity were excised from the gel and proteins were eluted, denatured and subjected to immunoblot analysis with a calpain 10 specific antibody. Mitochondrial and cytosolic fractions from primary cultures of renal proximal tubular cells (RPTC) were used as positive controls for the identification of calpain 10 splice variants, revealing the presence of the 75 kDa, 56 kDa, and 50 kDa (fig 2). Excised band A from the zymogram yielded the 56 kDa and the 50 kDa splice variants of calpain 10. Excised bands B and C from the zymogram contained the 75 kDa, 56 kDa and the 50 kDa splice variants. These results reveal that rabbit renal cortical mitochondrial matrix calpain activity is the result of one or more of the three calpain 10 splice variants.

Next we investigated calpain activity in mouse kidney mitochondria using zymography as described above. Two major bands of activity that did not co-migrate with cytosolic calpain 1 were identified (fig 3.). Interestingly, the bands from mouse mitochondria did not co-migrate with the bands from rabbit matrix, with the exception that band B in mouse mitochondria migrates close to band B in the rabbit matrix. Bands of activity were excised and subjected to immunoblot analysis as described above. Excised bands A and B from the mouse zymogram yielded the 75 kDa (runs slightly lower than the control), 56 kDa, and the 50 kDa splice variants of calpain 10 (fig 3). These results reveal that mouse renal mitochondrial calpain activity is the result of one or more of the three calpain 10 splice variants.

Rat kidney mitochondrial proteins were subjected to zymography as described above and calpain activity examined. Three major bands of activity, that did not co-migrate with purified cytosolic calpain 1, were identified (fig 4). These bands of activity did not co-migrate with any of the bands found in the rabbit matrix. Bands of activity from the rat zymogram were excised and subjected to immunoblot analysis. Excised band A from the rat zymogram yielded the 75 kDa (runs slightly lower than control) and the 50 kDa splice variants of calpain 10 (fig 4). Excised band B from the zymogram yielded the 56 kDa and the 50 kDa calpain 10 splice variants. Excised band C from the zymogram yielded the 75 kDa (runs slightly lower than control), the 56 kDa, and the 50 kDa splice variants. These results reveal that rat renal mitochondria calpain activity is the result of one or more of the three calpain 10 splice variants.

Taken together, these results reveal that multiple splice variants of calpain 10 are expressed and proteolytically active in renal mitochondria across three species. Interestingly, mitochondrial calpain 10 in each species seems to display a unique pattern of migration in native gels, while the migration patterns of denatured mitochondrial calpain 10 are similar. A number of reasons may contribute to this observation. For example, differences in amino acid composition, post-translational modifications, autolytic activation, or protein binding partners may alter calpain 10 migration in native gels. Further studies are needed to explore these possibilities.

Ideally, one would expect to see one band of activity from the zymogram be composed of one splice variant of calpain 10; however this was not the case. Multiple splice variants are present in each band of activity from the zymogram. Protein separation in native gel electrophoresis is dependent on the charge and shape of the protein, as well as its size [27], and therefore a 50 kDa and a 75 kDa calpain 10 splice variant may reasonably co-migrate. As noted above, protein binding partners also could explain this finding. In this case, each calpain 10 splice variant could independently bind to a common binding partner that imparts similar shape and charge on each splice variant, resulting in co-migration. Post-translational modifications also could influence charge characteristics such that separate splice variants would have similar native electrophoretic mobility.

We have identified three splice variants of calpain 10 using a calpain 10 specific shRNA knockdown approach. Kidney mitochondria from three species contained mitochondrial calpain activity that was inhibited by the calpain inhibitor, calpeptin. All sources of mitochondrial calpain activity are immunoreactive with calpain 10 antibodies, revealing that kidney mitochondria contain at least 3 splice variants of calpain 10 (75 kDa, 56 kDa, and 50 kDa). Additional studies are needed to elucidate the function and regulation of the calpain 10 splice variants.

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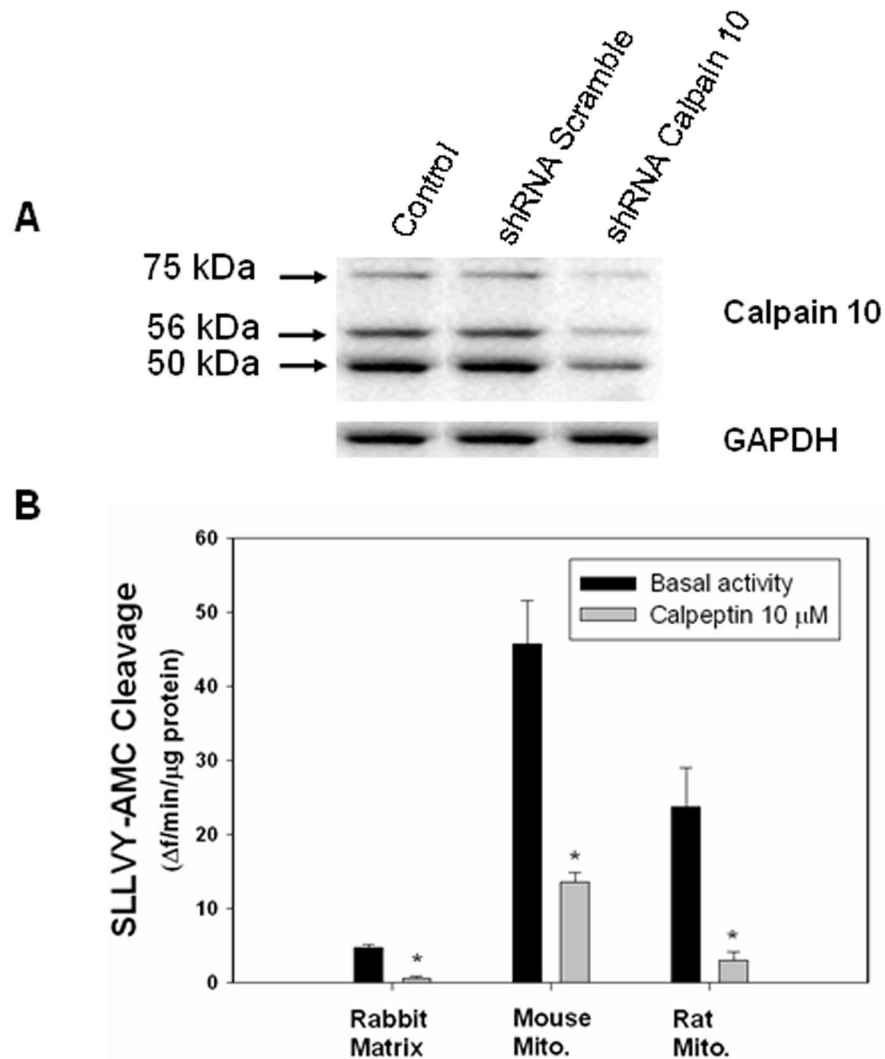
Figure 1. shRNA knockdown of Calpain 10 in NRK-52E cells and mitochondrial calpain activity
A) NRK52-E cells were transfected with shRNA constructs targeted to calpain 10 or scramble, and lysed after 48 h. Cell proteins were separated by SDS-PAGE and subjected to immunoblot analysis for CAPN10 and GAPDH. Representative of at least 3 experiments. **B)** Renal mitochondria from rabbit, mouse and rat were assayed for calpain activity using the fluorogenic calpain substrate SLLVY-AMC (50 μM) in the presence and absence of calpeptin. Data are expressed as means ± SE, n=3. Asterisks indicate means that are statistically significant from basal activity, $p<0.05$.
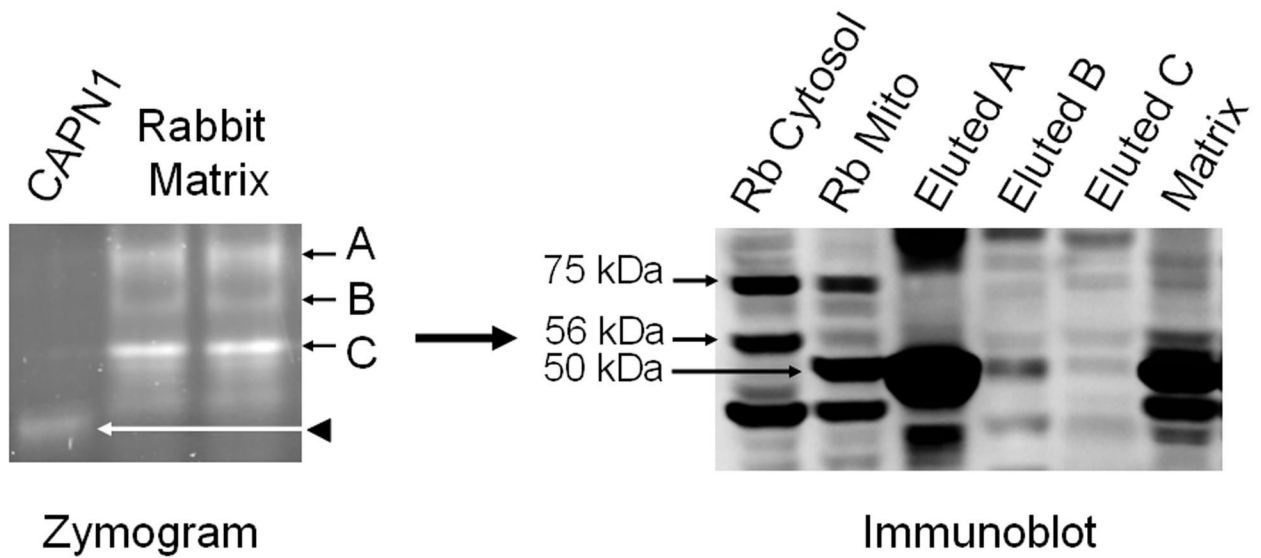
Figure 2. Rabbit renal cortical mitochondrial matrix contains multiple calpain 10 splice variants
Rabbit renal cortical mitochondrial matrix was separated on native-PAGE zymography gels. In matrix, three bands of activity (A, B, and C) were excised from the zymogram, and proteins eluted, separated by SDS-PAGE and subjected to immunoblot analysis for calpain 10. Rabbit RPTC cytosolic and mitochondrial fractions were used as positive controls for calpain 10 splice variants, 75 kDa (full length), 56 kDa, and 50 kDa. Lanes A, B, and C (immunoblot) correspond to the bands of activity A, B, and C from the zymogram. Representative of at least 3 experiments.
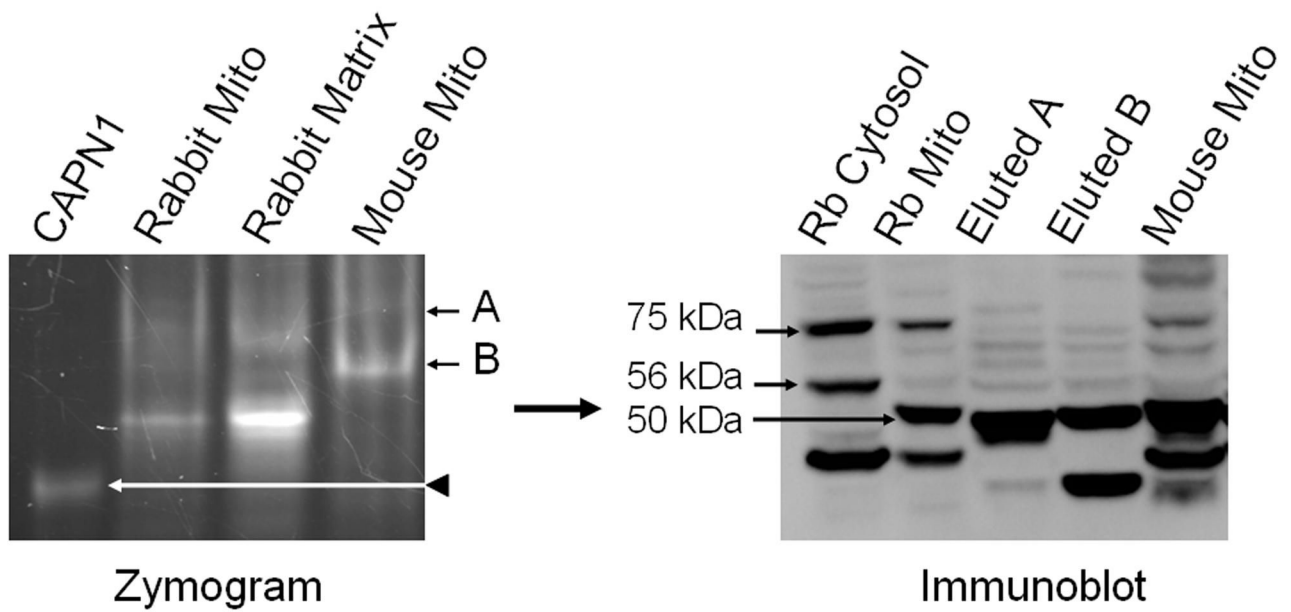
Figure 3. Mouse renal mitochondria contain multiple calpain 10 splice variants
Mouse renal mitochondria were separated on native-PAGE zymography gels. Two bands of activity (A and B) were excised from the zymogram, and proteins eluted, separated by SDS-PAGE and subjected to immunoblot analysis for CAPN10. Rabbit RPTC cytosolic and mitochondrial fractions were used as positive controls for calpain 10 splice variants, 75 kDa (full length), 56 kDa, and 50 kDa. Lanes A and B (immunoblot) correspond to the bands of activity (A and B) from the zymogram. Representative of at least 3 experiments.
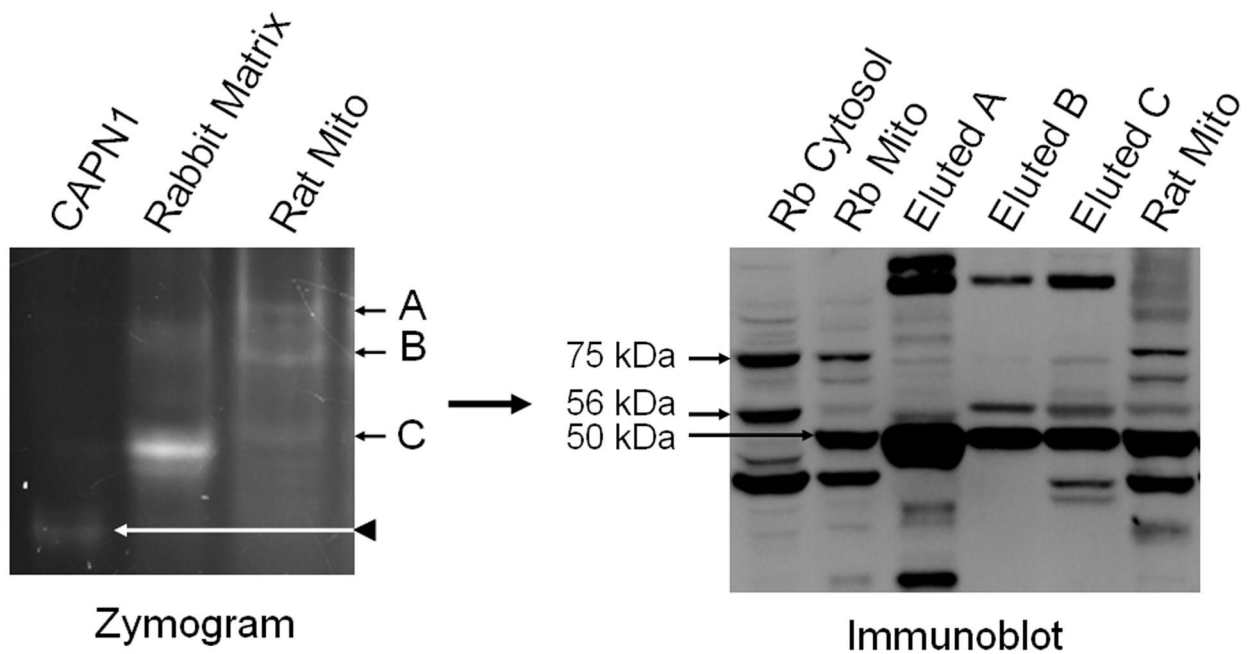
Figure 4. Rat renal mitochondria contain multiple calpain 10 splice variants
Rat renal mitochondria were separated on native-PAGE zymography gels. Three bands of
activity (A, B, and C) were excised from the zymogram, and proteins eluted, separated by SDS-
PAGE and subjected to immunoblot analysis for CAPN10 Rabbit RPTC cytosolic and
mitochondrial fractions were used as positive controls for calpain 10 splice variants, 75 kDa
(full length), 56 kDa, and 50 kDa. Lanes A, B, and C (immunoblot) correspond to the bands
of activity (A, B, and C) from the zymogram. Representative of at least 3 experiments.