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Research paper

Characterization of endogenous and recombinant human calpain-10

Biao Dong, Rihe Liu*

School of Pharmacy and Carolina Center for Genome Sciences, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

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Abstract

Calpain-10 is a novel ubiquitous calpain family member that has been implicated as a susceptibility gene for type 2 diabetes. One of the major challenges is that the function of calpain-10 is not yet known. To address this problem, we purified human calpain-10 from different sources, including the endogenous and the recombinant calpain-10 from HeLa S3 and 293F cells, respectively. Both endogenous and recombinant calpain-10 were present as two major forms with different origins. Interestingly, radiolabeled calpain-10 was found to be efficiently cleaved at the N-terminal region by calpain-2, but not by other proteases. None of these calpain-10 proteins have putative proteolytic activity under *in vitro* conditions when examined using different peptide substrates, including more than 70 *in vitro* translated, radiolabeled oligopeptides. Our results raise the possibility that calpain-10 may require a special intracellular localization or interacting partner(s) to acquire proteolytic activity, or it functions by interacting with other proteins rather than through its proteolytic activity.

Keywords: Calpain-10; Susceptibility gene for diabetes; Calpain-10 isoforms; Proteolytic activity; Radiolabeled oligopeptides; Random peptide library

1. Introduction

Calpain-10 is a new ubiquitous calpain family member that has been recently identified as the first candidate susceptibility gene for type 2 diabetes [1–5]. Since the first implication that genetic variations in the *CAPN10* gene are linked with increased risk of type 2 diabetes, many follow-up studies have been performed to verify the reported association in a number of ethnic populations. Intriguingly, varying levels of association between *CAPN10* and type 2 diabetes were reported. It appears that there is more than one *CAPN10* variant that could play a role in determining disease susceptibility, and the importance of different variants varies in different ethnic populations [5]. Due to these controversial results, functional studies on *CAPN10* in diabetes-related signaling pathways have drawn lots of attention. It was found that different variations in *CAPN10* were located in introns and could be associated with the change of expression level of *CAPN10* mRNA [1, 2]. In addition, these variations are also associated with diabetes related metabolic phenotypes, including free fatty acid level, insulin resistance, elevated triglyceride level, enhanced microvascular function, reduced β(3)-adrenoceptor function in fat cells, blood glucose level, and polycystic ovary syndrome [6–11]. Recently, several new findings suggested that calpain-10 protein itself could be involved in insulin secretion and action. It was reported that calpain-10 might facilitate GLU4 vesicle translocation during insulin stimulated glucose uptake in adipocytes [12]. Furthermore, it was found that a short-term exposure to the cell-permeable calpain inhibitors increased the insulin secretory response to glucose in mouse

Abbreviations: ALLN, calpain inhibitor Ac-Leu-Leu-Nle-H; *CAPN1*, calpain-1 gene; *CAPN10*, calpain-10 gene; DMED, Dulbecco's Modified Eagle's Medium; GLU4, glucose transporter member 4; NDUFV2, NADH dehydrogenase (ubiquinone) flavoprotein 2; ND6, NADH dehydrogenase subunit 6; NEAA, non-essential amino acids; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; PBS, phosphate- buffered saline; SDS, sodium dodecyl sulfate; SNARE, soluble NSF attachment receptor; SNAP-25, synaptosome-associated protein of 25 kDa; TBS, Tris buffered saline; TMV, tobacco mosaic virus; TNT, coupled *in vitro* transcription and translation; UPS, ubiquitin-proteasome system; UTR, untranslated region.

* Corresponding author. Tel.: +1 919 843 3635; fax: +1 919 968 0081.
E-mail address: rliu@email.unc.edu (R. Liu).

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pancreatic islets [13], whereas a 48-h long-term exposure of mouse islets to calpain inhibitors reversibly suppressed glucose-induced insulin secretion [14].

One critical question that remains to be addressed is whether this protease domain-containing protein has the putative proteolytic activity? Two recent investigations provided important clues. First, a 54 kDa isoform of calpain-10 was found to directly interact with t-SNAREs, syntaxin 1, and SNAP-25 in pancreatic beta cells [15]. Significantly, SNAP-25 was found to undergo a Ca^{2+}-dependent partial proteolysis during stimulated secretion, raising the possibility that calpain-10 regulates insulin secretion through proteolytic cleavage of SNAP-25. More recently, two complex I proteins, namely NDUFV2 and ND6, were implicated as the potential downstream substrates of calpain-10 in mitochondria [16]. In all these studies, the observed cleavage was indirectly attributed to calpain-10, presumably because the cleavage could be abolished by general calpain inhibitors. To biochemically characterize this protein, we purified calpain-10 from different sources, including the endogenous and the recombinant calpain-10 from HeLa S3 and 293F cells, respectively. Both endogenous and recombinant calpain-10 were present as two major forms, but with very different origins. We demonstrate that radiolabeled calpain-10 could be efficiently cleaved at the N-terminal region by calpain-2. However, none of these calpain-10 proteins has putative proteolytic activity when used for direct proteolytic analysis against various general fluorogenic calpain substrates and more than 70 *in vitro* translated, radiolabeled oligopeptides. Our results raise the possibility that calpain-10 requires special intracellular localization or other interacting partner(s) to acquire proteolytic activity, or it functions by interacting with other proteins rather than through its proteolytic activity.

2. Materials and methods

2.1. Cell lines

Insect Sf9 cells from Novagen (Madison, WI) were grown at 27 °C as a semi-adherent culture in BacVector insect cell medium (Novagen). HeLa S3 cells from UNC tissue culture facility were maintained at 37 °C in a humidified incubator with 5% CO_2 and grown to the stationary phase in Ham's F12 medium supplemented with 10% fetal bovine serum. Human kidney 293F cells from Invitrogen (Carlsbad, CA) were cultured in a high glucose DMED medium with 1× NEAA, 2 mM glutamine, 10% fetal bovine serum, and 1× penicillin/streptomycin.

2.2. Cloning of the full-length human CAPN10a gene into different expression vectors

A cDNA clone (MGC-10770) containing the full-length human *CAPN10a* gene was obtained from ATCC (Manassas, VA) and used as the template for PCR amplification. To clone *CAPN10a* for expression in insect cells, the full-length coding sequence was PCR amplified using a high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) and inserted into expression vector pIEx-1 Ek/LIC with N-terminal His×6- and S-tags according to the manufacturer's instructions (Novagen). To clone *CAPN10a* for expression in mammalian cells, the amplified coding sequence was inserted into expression vector pcDNA3.1D/V5-His TOPO according to the manufacturer's instructions (Invitrogen). The resulting *CAPN10a*-containing expression vectors with correct sequences were selected and confirmed by sequencing analysis.

2.3. Overexpression of calpain-10 in transfected Sf9 insect cells

Sf9 cells were transfected with pIEx-*CAPN10a* using Insect GeneJuice transfection reagent according to the manufacturer's instructions (Novagen). The cells were harvested at 48 h after transfection and washed twice with ice-cold PBS, followed by cell lysis on ice in a buffer containing 50 mM Tris–HCl at pH 7.4, 150 mM NaCl, and 1% Triton X-100. After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was collected and the amount of total protein quantified. To detect the overexpressed calpain-10 present in the membranes, the insoluble fraction was dissolved in an SDS sample buffer (100 mM Tris–HCl at pH 6.8, 2% SDS, 0.01% bromophenol blue, 5 mM β-mercaptoethanol, and 10% glycerol) by heating at 95 °C for 5 min. The presence of recombinant human calpain-10 was detected by probing the Western blots using an anti-S tag antibody (Novagen) at 1:2000 dilution.

2.4. Overexpression and purification of calpain-10 in stably transfected 293F cells

293F cells were transfected with pcDNA3.1D/V5-His-*CAPN10a* using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Selection of the stably transfected cell lines was initiated at 24 h after transfection using a culture medium containing 750 μg/ml G418. The selection was accomplished during a period of 30 days and G418-resistant cell lines were maintained in the selective culture medium.

To purify overexpressed calpain-10a, stably transfected cells from 0.5 L of culture were lysed in 12 ml of lysis buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1% Triton X-100) on ice for 30 min, followed by centrifugation at 14,000 rpm for 20 min. The supernatant was mixed with 0.6 ml of Ni-NTA agarose beads (Qiagen, Valencia, CA) at 4 °C for 2 h and the mixture loaded into an empty Bio-Rad column for separation. After washing the column three times with 15 ml of wash buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 20 mM imidazole, and 0.05% Tween 20), the bound protein was eluted using 3.0 ml of elution buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 250 mM imidazole, and 0.05% Tween 20). The eluate was dialyzed in a buffer containing 10 mM Tris–HCl at pH 7.4, 50 mM NaCl, 1 mM EDTA, and 0.05% Tween 20. All purification steps were performed at 4 °C. Calpain-10 thus purified was