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Discrete proteolysis of neuronal calcium sensor-1 (NCS-1) by μ-calpain disrupts calcium binding

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ABSTRACT

Neuronal calcium sensor-1 (NCS-1) is a high-affinity, low-capacity Ca^{2+}-binding protein expressed in many cell types. We previously showed that NCS-1 interacts with inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and modulates Ca^{2+}-signaling by enhancing InsP3-dependent $InsP_3R$ channel activity and intracellular Ca^{2+} transients. Recently we reported that the chemotherapeutic agent, paclitaxel (taxol) triggers μ-calpain dependent proteolysis of NCS-1, leading to reduced Ca^{2+}-signaling within the cell. Degradation of NCS-1 may be critical in the induction of peripheral neuropathy associated with taxol treatment for breast and ovarian cancer. To begin to design strategies to protect NCS-1, we treated NCS-1 with μ-calpain *in vitro* and identified the cleavage site by N-terminal sequencing and MALDI mass spectroscopy. μ-Calpain cleavage of NCS-1 occurs within an N-terminal pseudoEF-hand domain, which by sequence analysis appears to be unable to bind Ca^{2+}. Our results suggest a role for this pseudoEF-hand in stabilizing the three functional EF-hands within NCS-1. Using isothermal titration calorimetry (ITC) we found that loss of the pseudoEF-hand markedly decreased NCS-1's affinity for Ca^{2+}. Physiologically, this significant decrease in Ca^{2+} affinity may render NCS-1 incapable of responding to changes in Ca^{2+} levels *in vivo*. The reduced ability of μ-calpain treated NCS-1 to bind Ca^{2+} may explain the altered Ca^{2+} signaling in the presence of taxol and suggests a strategy for therapeutic intervention of peripheral neuropathy in cancer patients undergoing taxol treatment.

1. Introduction

Neuronal calcium sensor-1 (NCS-1) is a calcium (Ca^{2+}) binding protein important in intracellular signaling. NCS-1 is composed of four 'helix–loop–helix' EF-hand motifs, an ancestral structural EF-hand domain (pseudoEF-hand) which has lost the ability to bind Ca^{2+} and three functional EF-hands (EF-hand 1, 2 and 3) which bind Ca^{2+} with varying affinities [1,2]. Although structurally nearly identical to EF-hands 1,2, and 3, the pseudoEF-hand is not a functional Ca^{2+}-binding site, and is unable to bind Ca^{2+} due to a lack of acidic amino acids (Asp or Glu) at the +X and −Z positions in the loop, which are required for Ca^{2+} coordination [1,2]. Interaction of NCS-1 with downstream proteins is regulated by Ca^{2+} binding and N-terminal myristolation. Although both Ca^{2+} binding and myristolation induce conformational changes, myristolation is not required for the interaction between NCS-1 and the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) [3]. The binding of NCS-1 to the $InsP_3R$ enhances Ca^{2+} signaling [3].

Recently, we found that the chemotherapeutic drug paclitaxel (taxol) binds to NCS-1 and addition of taxol to cells further enhances the NCS-1 amplification of $InsP_3R$-dependent Ca^{2+} signaling [4]. We have shown that taxol in nanomolar concentrations induced oscillatory changes in cytosolic Ca^{2+} in an $InsP_3R$-dependent manner, and increased binding of NCS-1 to the $InsP_3R$, whereas knockdown of NCS-1 abrogated taxol-induced Ca^{2+} oscillations. In addition, taxol at a concentration between 80 and 800 ng/ml (937 nM) is sufficient to induce Ca^{2+} oscillations, and taxol binds to NCS-1 with an EC_{50} of 728 ± 44 ng/ml (557 ± 34 nM), all within the range observed in taxol-treated patients (steady-state plasma concentrations in patients treated with taxol are between 85 and 850 ng/ml) [4]. The effects of taxol on Ca^{2+} signaling are potentially important because it is a drug used to treat a variety of tumor types including ovarian, breast, lung, head, and neck cancers (reviewed by [5]). Although it is clear that taxol exerts its chemotherapeutic effect through its action on microtubule assembly [6], taxol also induces an irreversible peripheral neuropathy in over 30% of treated individuals and the mechanism of this side effect is unclear [7]. Dis-

Abbreviations: NCS-1, neuronal calcium sensor-1; ITC, isothermal titration calorimetry; Ca^{2+}, calcium; MALDI-MS, matrix assisted laser desorption/ionization mass spectroscopy.

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turbed homeostasis of Ca^{2+} has been proposed as the cause of the taxol-induced peripheral neuropathy [8,9]. In mouse models, pretreatment with μ-calpain antagonists abrogated the taxol-induced peripheral neuropathy [10].

In isolated cells, the immediate response to addition of taxol is the appearance of $InsP_3$-mediated Ca^{2+} oscillations [4]. Exposure of cells to taxol for several hours, which more closely approximates the situation when taxol is used as a chemotherapeutic agent, abolishes $InsP_3$-mediated Ca^{2+} signaling [11]. The sequence of events appears to be an immediate enhancement of Ca^{2+} release from intracellular stores which activates μ-calpain. In particular, μ-calpain activity was significantly higher in taxol-treated cells (800 ng/ml, 6 h) than vehicle-treated cells, using concentrations of taxol within the therapeutic range [11]. The degradation of NCS-1 follows the activation of μ-calpain and the subsequent loss of NCS-1 leads to the attenuation of $InsP_3$-mediated Ca^{2+} signaling [11]. The loss of NCS-1 is believed to result in a negative-feedback loop, leading to the cessation of Ca^{2+} oscillations and impaired phosphoinositide-mediated Ca^{2+} signaling [11] Taxol administration to mice also leads to decreased NCS-1 levels [11]. NCS-1 levels can be maintained in cells when inhibitors of μ-calpain are included [11].

In this study we show that specific proteolysis of NCS-1 by μ-calpain can occur *in vitro*. The location of the cleavage site alters the pseudoEF-hand site and the ability to bind Ca^{2+} is diminished. From structural considerations this cleavage could induce changes in exposed hydrophobic surface areas that could alter the specificity of protein–protein interactions. In this case, specific cleavage of NCS-1 by μ-calpain appears to create an altered NCS-1 protein with deficient Ca^{2+} binding properties. The altered Ca^{2+} binding will attenuate $InsP_3R$-dependent Ca^{2+} signaling.

2. Experimental methods

2.1. Overexpression of NCS-1

NCS-1 was produced by overexpressing rat NCS-1 cDNA subcloned into pET21-a+ bacterial expression vector (provided by Andreas Jeromin, Baylor College). NCS-1 purification protocol was modified from that described by Zozulya et al. [12]. The NCS-1 vector was transformed into Statagene BL21(DE3) Codon Plus RIL competent *E. coli* cells. Cells were grown at 37 °C in 2 L baffled flasks with 1 L LB broth and ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL). At an OD_{595nm} of 0.5–0.7, overexpression was induced with 0.5 mM isopropyl-D-thiogalactoside (IPTG) and cells were shifted to 18 °C for ~16 h. Cells were harvested by centrifugation (3000 rpm, 30 min, 4 °C) and resuspended in 10 mL of 50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

2.2. Protein purification by hydrophobic interaction chromatography

Bacteria expressing recombinant NCS-1 were lysed with lysozyme (Sigma, 2 mg/mL) coupled with 3 freeze–thaw cycles using ethanol-dry ice. Cell lysate was homogenized by tip sonication (Branson Sonicator) for 2 min on ice using a 50% duty cycle. Homogenized lysate was clarified by centrifugation at 40,000 × *g* (20,000 rpm, 1 h, 4 °C) and the resulting supernatant further sonicated for 2 min at 50% duty cycle to reduce sample viscosity. Hydrophobic interaction chromatography (HIC) was used to purify NCS-1 as described [13]. Large-scale purification of NCS-1 protein was performed using a GE Healthcare HiTrap Phenyl HP high substitution 5 mL column. The lysates were loaded on a column equilibrated in 50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, and 10 mM $CaCl_2$. Following sample application the column was washed with 10 column volumes of 50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, and 10 mM $CaCl_2$. Recombinant NCS-1 protein was eluted using 50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, and 50 mM EDTA in 25 × 1 mL fractions using eppendorf tubes on ice. The protein was eluted with 50 mM EDTA to improve yield. Protein purification was monitored using SDS-PAGE. 1 mL elution fractions were collected (fractions 5–7 of 25 mLs) and pooled. The concentration of NCS-1 containing fractions (typically fractions 5–7 of 25 mLs) was determined using the Bradford Assay (Bio-Rad). Elution fractions were pooled if above A_{595nm} = 0.200. Yields of 10–15 mg of pure NCS-1 are obtained using the above procedure.

2.3. Preparation of Ca^{2+}-free protein

Purified recombinant NCS-1 was stripped of Ca^{2+} using a method modified from Fisher et al. [13]. Briefly, protein was buffer exchanged using a Bio-Rad Econo-Pac 10DG column equilibrated in 50 mM HEPES, 100 mM KCl, at pH 7.5. NCS-1 was then dialyzed against 10 mM EDTA at pH 2.0 in a Pierce Slide-A-Lyzer 7k dialysis cassette for 1 h, followed by dialysis against Millipore deionized water for 1 h, and then 10 mM Tris pH 7.4 for 1 h. Last, the protein was dialyzed against 50 mM HEPES, pH 7.5, containing 100 mM KCl, 1 mM DTT. Dialysis was done using all plastic containers. The protein was concentrated (up to 2.14 mg/mL or 100 μM) using a Millipore Ultracel – 10 K Amicon Ultra-15 centrifugal filter device.

2.4. Digestion of NCS-1 by μ-calpain

We used μ-calpain was purchased from SIGMA (Calpain-1, Active From Human Plasma SIGMA C-6108) and was >98% by SDS-PAGE according to the manufacturer. NCS-1 in vitro proteolysis trials were also conducted with Calpain-1 from Human Erythrocytes (Calbiochem Cat# 208713) at >95% purity by SDS-PAGE with similar results (data not shown). The digestion of NCS-1 produced discrete cutting of the protein using 1:5 and 1:10 mass ratio of μ-calpain to NCS-1. The reaction volume was 14.9 μL. The digestion buffer was 10 mM HEPES, 10 mM DTT, 5 mM Ca^{2+}, and 3.5 mM EDTA at pH 7.2 [4]. Reactions contained 10 μL (0.7 mg/mL) NCS-1, 7 μg total, or buffer for controls. The 1:5 reaction contained 1.4 μL of μ-calpain at a concentration of 1 mg/mL. The 1:10 reaction contained 1.4 μL of μ-calpain at a concentration of 0.5 mg/mL. Control reactions included undigested NCS-1 and μ-calpain without NCS-1 incubated with the digestion reactions. Immediately upon addition of μ-calpain the reaction was incubated at 37 °C for 10 min. The reaction was quenched on ice after the addition of 14.9 μL SDS-PAGE buffer.

2.5. SDS-PAGE gel electrophoresis, N-terminal sequencing of NCS-1 digestion products

SDS-PAGE of undigested and digested NCS-1 was performed on 15% polyacrylamide. The proteins were visualized using Invitrogen SimplyBlue SafeStain and scanned into digital image format for analysis. 1:5 and 1:10 NCS-1 μ-calpain cleavage reaction products were prepared. Approximately 7 μg of digested protein was loaded in each lane. Protein was transferred to PVDF membrane, Immobilon-P^{SQ}, for N-terminal sequencing using a wet western blot transfer apparatus and 15% SDS-PAGE at 250 mA for 1 h. The PVDF membrane was rinsed in ddH_2O and stained with coomassie brilliant blue. The coomassie blue stained bands of interest at 17.5 kDa and 13 kDa were cut out from the membrane and sent for N-terminal sequencing by Edman degradation at the Tufts Core Facility (Tufts Medical School, Boston, MA). The Tufts Core Facility uses an ABI 494 protein sequencer.