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Glycosylation controls sodium-calcium exchanger 3 sub-cellular localization during cell cycle

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ABSTRACT

The Na^+/Ca^{2+} exchanger (NCX) is a membrane antiporter that has been identified in the plasma membrane, the inner membrane of the nuclear envelope and in the membrane of the endoplasmic reticulum (ER). In humans, three genes have been identified, encoding unique NCX proteins. Although extensively studied, the NCX's sub-cellular localization and mechanisms regulating the activity of different subtypes are still ambiguous. Here we investigated the subcellular localization of the NCX subtype 3 (NCX3) and its impact on the cell cycle. Two phenotypes, switching from one to the other during the cell cycle, were detected. One phenotype was NCX3 in the plasma membrane during S and M phase, and the other was NCX3 in the ER membrane during resting and interphase. Glycosylation of NCX3 at the N45 site was required for targeting the protein to the plasma membrane, and the N45 site functioned as an on-off switch for the translocation of NCX3 to either the plasma membrane or the membrane of the ER. Introduction of an *N*-glycosylation deficient NCX3 mutant led to an arrest of cells in the G0/G1 phase of the cell cycle. This was accompanied by accumulation of de-glycosylated NCX3 in the cytosol (that is in the ER), where it transported calcium ions (Ca^{2+}) from the cytosol to the ER. These results, obtained in transfected HEK293T and HeLa and confirmed endogenously in SH-SY5Y cells, suggest that cells can use a dynamic Ca^{2+} signaling toolkit in which the NCX3 sub-cellular localization changes in synchrony with the cell cycle.

1. Introduction

The calcium ion (Ca^{2+}) is an intracellular second messenger that plays a crucial role in many biological processes (Berridge et al., 2000). The basal cytosolic concentration of Ca^{2+} is low (100 nM) and an accurate control of the Ca^{2+} homeostasis is essential for cell life, since a high level of Ca^{2+} is toxic to the cell. This is mainly accomplished by a variety of Ca^{2+}-ATPases (the plasma membrane Ca^{2+} pump (PMCA) and sarco/endo-plasmic reticulum Ca^{2+}-ATPases (SERCA)), the mitochondrial Ca^{2+} uniporter, and the Na^+/Ca^{2+} exchanger (NCX) (Hilge et al., 2006; Uhlen and Fritz, 2010).

NCX, as a plasma membrane antiporter, is important for modulating the intracellular Ca^{2+} homeostasis by coupling Ca^{2+} efflux to Na^+ influx (forward mode) and Na^+ efflux to Ca^{2+} influx (reverse mode), depending on the transmembrane ion gradient and membrane potential (Iwamoto, 2007). The NCX has also been found in the inner membrane of the nuclear envelope (NE), where it is proposed to regulate Ca^{2+} flux between the nucleoplasm and the NE (Xie et al., 2002; Wu et al., 2009; Brini and Carafoli, 2011). In mammals, three NCX subtypes have been reported: NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996). They share ~70% sequence identity and show few functional differences (Iwamoto and Shigekawa, 1998; Linck et al., 1998; Hilge et al., 2006).

The NCX protein contains a cleaved signal sequence, a short glycosylated extracellular region (Hryshko et al., 1993), eleven transmembrane segments (TMS), and a large central cytoplasmic loop. The eleven TMSs can be grouped into an N-terminal hydrophobic domain, composed of the first five TMSs (1–5), and a C-terminal hydrophobic domain, composed of the last six TMSs (6–11). Two highly conserved regions, named alpha-1 and −2 repeats were identified in the protein (Schwarz and Benzer, 1997; Liao et al., 2012) and suggested to be involved in the Na^+ and Ca^{2+} binding (Iwamoto et al., 1999; Liao et al., 2012). The N-terminal hydrophobic domain is separated from the C-terminal hydrophobic domain through a large hydrophilic intracellular loop (Nicoll et al., 1999). Removal or cleavage of the loop results in an hyperactive exchanger and calcium refilling of the ER (Matsuoka et al., 1993; Michel et al., 2017). The exchanger inhibitory peptide (XIP) locates in the N-terminus of this loop, and the Ca^{2+} binding domains

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CBD1 and CBD2 plus an α-catenin-like domain constitute the Ca^{2+} binding function of the loop. CBD1 is the main sensor of calcium content and activates the exchanger in a Ca^{2+} transient state (Hilge et al., 2006), whereas the inactivation of NCX is regulated by CBD2 (Giladi and Khananshvili, 2013).

Glycosylation is a ubiquitous post-translational modification of proteins in animal systems playing a number of crucial roles from protein folding and quality control to biological recognition events (Moremen et al., 2012). It has been described that protein *N*-glycosylation plays a crucial role in controlling the number of voltage-gated calcium channels embedded in the plasma membrane and also in their functional gating properties (Lazniewska and Weiss, 2017). Although the NCX3 protein, as the key player in cytosolic calcium extrusion, has been localized at the junction of PM and ER/SR, the sub-cellular localization and trafficking of the NCX3 proteins have still not been described in detail.

In this study, we show that, in mammalian cells, the sub-cellular localization of the NCX3 protein varies between resting/interphase cells and cells in the cell cycle. Firstly, we can identify two primary phenotypes. One is NCX3 localized in the plasma membrane (NCX3-PM), the other is NCX3 distributed in the cytoplasm (NCX3-ER). Secondly, in interphase cells, NCX3 localizes to the ER and nuclear membrane, while during S-phase, mitosis and cell division it is observed in the plasma membrane. Intriguingly, the presence and role of *N*-glycosylation of human NCX3 has not previously been defined. Here we show that modification of NCX3 by *N*-linked glycosylation at a single asparagine residue, N45, is required for targeting of the protein to the plasma membrane. Importantly, this mechanism also affects cells' progression through the cell cycle.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's media (Thermo Fisher), supplemented with 10% fetal bovine serum and 1% Pen/Strep (Thermo Fisher). HeLa cells were grown in Eagle's Minimum Essential Medium (Thermo Fisher), supplemented with fetal bovine serum to a final concentration of 10%. Transient transfections with different plasmids were carried out using Lipofectamine Plus or Lipofectamine 2000 according to the manufacturer's instructions (Thermo Fisher). Stable expression cell lines were established following the protocol from Thermo Fisher.

2.2. Construction of plasmids and site-directed mutagenesis

The cDNA containing the entire open reading frame (ORF) of the human NCX3.2 isoform had previously been cloned into the pcDNA3.1 expression plasmid (Lindgren et al., 2005), generating a fusion of NCX3 cDNA with a C-terminal V5-His tag (pcDNA3.1-NCX3.2/V5-His). The plasmids pcDNA3.1-GFP and pcDNA3.1/V5-His were used as control plasmids.

The recombinant NCX3.2-EmGFP was attained by polymerase chain reaction (PCR). The EmGFP fragment was amplified by PCR using primers SP + GFP-F: 5′-GCT GGT GGC TCA GGG GAC ATG GTG AGC AAG −3′ and GFP + NCX3-R: 5′-CTG CCC TGT GCT TGG CAC CTT GTA CAG CTC-3′. Insertion of EmGFP into the NCX3.2 cDNA between the aspartic acid (D^{37}) and valine (V^{38}) required another two fragments. One was from the N-terminal to D^{37} using the primers NCX3.2-start: 5′-GCC GCC ACC ATG GCG TGG TTA AGG TTG-3′ and SP + GFP-R: 5′-CTC GCC CTT GCT CAC CAT GTC CCC TGA GCC-3′. The other was from V^{38} to the C-terminal end of NCX3.2 using the primers GFP + NCX3-F: 5′-ATG GAC GAG CTG TAC AAG FTF CCA CGC ACA-3′ and NCX3-orf-end-R: 5′-GAA CCC CTT GAT GTA GCA ATA GGC-3′. Subsequently, using the three fragments as templates, the NCX3.2-EmGFP was constructed by PCR with the primers NCX3.2-start: 5′-GCC GCC ACC ATG GCG TGG TTA AGG TTG-3′ and NCX3-orf-end-R: 5′-GAA CCC CTT GAT GTA GCA ATA GGC-3′. The NCX3.2-EmGFP was cloned into the pcDNA3.1-NCX3.2/V5-His TOPO vector according to the method described in the previous study (Lindgren et al., 2005).

Site directed mutagenesis was carried out using a protocol based on the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) using the plasmid (pcDNA3.1-NCX3.2/V5-His) with primers (mutated nucleotides are underlined) N45D-F: 5′- CAA GCA CAG GGC AGA ACG ATG AGT CCT GTT CAG GG-3′ and N45D-R: 5′- CCC TGA ACA GGA CTC ATC GTT CTG CCC TGT GCT TG-3′ for N45D mutation, and primers N67D-F: 5′-CAA TCT GGT ACC CGG AGG ACC CTT CCC TTG GGG AC-3′ and N67D-R: 5′-GTC CCC AAG GGA AGG GTC CTC CGG GTA CCA GAT TG-3′ for N67D mutation. In this way, the asparagine residues N45 and N67 in two putative *N*-linked glycosylation motifs of NCX3 (see Fig. 6D and F) were mutated to aspartic acid. The respective cDNA mutants and resulting proteins were designated as NCX3-N45D and NCX3-N67D. The introduced mutations were confirmed by sequencing.

2.3. RNA interference (RNAi) for gene silencing

Information on specific siRNAs used in this study is as follows: SLC8A3 Stealth Select RNAi™ siRNA (HSS143968), SLC8A3 Stealth Select RNAi™ siRNA (HSS143969) and SLC8A3 Stealth Select RNAi™ siRNA (HSS143970). The scrambled Stealth RNAi™ siRNA Negative Control Kit (12935-100) with similar GC content recommended by Invitrogen was used as control siRNA.

2.4. Measurement of $[Ca^{2+}]_i$ in HeLa cells using Fura-2

HeLa cells were cultured in 35 mm Petri dishes and transfected with plasmids as indicated in each experiment. After 24 h, cells were rinsed with Ca^{2+}-free Krebs buffer and loaded with 5 μM Fura-2/AM in 2 ml of the same buffer for 30 min at 37 °C in the dark. Subsequently, cells were rinsed with the buffer twice and the Petri dish was attached to a microscope stage and monitored with time-lapse microscopy. Measurements were carried out at 37 °C using a heat-controlled chamber (Warner Instruments) with a cooled electron-multiplying charged-coupled device Cascade II:512 camera (Photometrics) mounted on an inverted microscope (Carl Zeiss) equipped with a 25 × 0.8 NA. lens (Carl Zeiss). Excitation at 340 nm and 380 nm was assessed with a high-speed illumination system (DG-4, Sutter Instrument). MetaFluor (Molecular Devices) was used to control all devices and to analyze acquired images. Thapsigargin (1 μM) was added for releasing Ca^{2+} from ER into cytosol. The area under the curve was calculated to compare responses.

2.5. SDS-PAGE and Western blotting

Transfected cells were pelleted and dissolved in SDS-sample loading buffer (0.125 M Tris, 15% (v/v) glycerol, 5 mM EDTA, 2% SDS, 0.1% (w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol, pH6.8) prior to a 10 min incubation at 100 °C. Samples were separated on a 10% polyacrylamide gel, transferred to PVDF (Immobilon P, Millipore), and probed using a monoclonal anti-V5 antibody (1:5000, Thermo Fisher). Subsequently, the blots were incubated with an anti-mouse secondary antibody conjugated to horse-radish peroxidase (Amersham), and immunocomplexes were detected with enhanced chemiluminescence (ECL) (Amersham). In some experiments, transfected HEK293T cells were treated (at 12 h post-transfection) with and without tunicamycin (an inhibitor of *N*-linked glycosylation, Sigma) for an additional 20 h followed by immunoblotting analysis.

2.6. Indirect immunofluorescence

The HEK293T and HeLa cells were seeded onto glass coverslips after one day, transfected with plasmids, and were fixed (4%

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