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Functional and Physical Interaction between Human Lactate Dehydrogenase B and Na^+/H^+ Exchanger Isoform 1

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Abstract: The ubiquitous plasma membrane Na^+/H^+ exchanger 1 (NHE1) is rapidly activated in response to various extracellular stimuli and maintains normal cytoplasmic pH. Yeast two-hybrid screening was used in order to identify proteins interacting with NHE1 using its cytoplasmic domain as a bait from HeLa cDNA library. One of the interacting cDNA clones was human Lactate dehydrogenase B (LDHB). *In vitro* translated LDHB was pulled down together with GST-NHE1.cd protein in the GST pull down assay, confirming the interaction *in vitro*. LDHB antibody immunoprecipitated endogenous LDHB together with NHE1 from H9c2 cells, validating cellular interaction between NHE1 and LDHB. Subsequent analysis revealed that the overexpression of LDHB increased intracellular PH, implying opening of the NHE1 transporter. Moreover, overexpression of LDHB activated caspase 3 and induced cell death, consistent with the expected phenotype of hyper-activation of NHE1. Collectively, our data indicate that LDHB modulates NHE1 activity via physical interaction.

Keywords: NHE1, LDHB, pHi, caspase 3, cell death

INTRODUCTION

The steady-state condition of intracellular pH during physiological conditions requires the continuous cellular export of acidic equivalents. Na^+/H^+ exchanger (NHE) removes one intracellular H^+ ion for an extracellular Na^+ ion, resulting in a stable intracellular pH (Noël and Pouysségur, 1995). The nine NHE isoforms (NHE1-9) were identified in various mammalian cells. NHE1 was an important regulator of cell volume, cell migration, adhesion, shape determination, proliferation, and cell death. And NHE1 responds to pathophysiological stresses such as ischemia, osmotic stress, acidosis, and mechanical stress (Putney et al., 2002; Luo and Sun, 2007).

NHE1 molecule harbors 12 N-terminal transmembrane domains and a C-terminal cytoplasmic regulatory domain. NHE1 could directly interact with multiple binding partners. NHE1 acts as a scaffold protein in response to the signalling events. There exists a long list of NHE1-interacting proteins. The partners are 14-3-3, HSP70, Bin1, Daxx, tescalcin, carbonic anhydrase, Ca^{2+}/calmodulin, members of the calcineurin B homologous protein (CHP) family, phosphatidylinositol 4,5-bisphosphate (PIP_2), ezrin, radixin, and moesin (Lehoux et al., 2001; Mailänder et al., 2001; Xue et al., 2007; Jung et al., 2008; Zaun et al., 2008). Diverse physiological effects by NHE1 may be derived from such broad interactions, rather than simply facilitating H^+ ion fluxes (Baumgartner et al., 2004; Pedersen, 2006). The C-terminal region of NHE1 is phosphorylated by diverse serine/threonine protein kinases, including ROCK1, p90RSK, Nck-interacting kinase (NIK), PKA, PKC, p160ROCK, ERK1/2, and p38 (Fliegel and Fröhlich, 1993; Tominaga et al., 1998; Takahashi et al., 1999; Khaled et al., 2001; Yan et al., 2001; Malo et al., 2007). Conversely, PP1 and PP2A act as negative regulators of NHE1.

Lactate dehydrogenase (LDH; EC 1.1.1.27) is a tetrameric enzyme composed of two subunits (LDHA and LDHB) (Markert, 1963). LDH catalyses the interconversion of pyruvate and lactic acid with concomitant interconversion of NADH and NAD^+. The four LDH isozymes (LDH A-D) are expressed in a tissue dependent manner. The Human LDH isozymes (LDH A-C) have 84-89% sequence similarities and 69-75% amino acid identities (Millan et al., 1987). LDHA was suited for pyruvate reduction in muscle tissues (Markert et al., 1975), whereas LDHB, for lactate oxidation in heart tissue (Maekawa, 1988). LDHC was present in testis (Edwards et al., 1987). LDHD encodes for mitochondrial protein (Flick and Konieczny, 2002). The

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changes in serum LDH isoenzyme pattern are linked to cancer development (Chen et al., 2006).

In the present study, we performed yeast two-hybrid analysis and isolated LDHB as a novel binding partner of NHE1. The interaction between LDHB and NHE1 was validated *in vitro* and in H9c2 cells. The physical interaction promoted NHE1 activation, leading to cell death.

MATERIALS AND METHODS

Plasmid Constructions

Preparation of the constructs LexA/NHE1.cd, LexA/FADD, pJG4-5/TRADD, pJG4-5/Daxx, pJG4-5/LDHB, and pGEX-4T-1/NHE1.cd are described previously (Ryu et al., 2000; Jung et al., 2008). pCNS/LDHB was supplied from the 21C Frontler Human Gene Bank (Korea).

Yeast transformation and β-galactosidase assay

pLexA/NHE1.cd bait and B42 activation domain (AD)-human HeLa cDNA library (Clontech, Palo Alto, CA) were cotrasformed into the EGY48 strain and plated on synthetic drop-out agar deficient in leucine, tryptophan and histidine. In general, colonies grew in 3-6 days, and were screened for β-galactosidase activity. Strongly interacting clones were isolated from the library plasmids and retransformed into the reporter strain to examine for interactions with NHE1.cd, along with positive or negative control baits.

Cell Culture and DNA Transfection

The embryonic rat heart-derived myogenic H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin/streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. H9c2 cells were transfected using the nucleofection system from Amaxa (Germany). 1×10^6 cells were suspended in 100 μL Nucleofector Solution Kit R (Amaxa) containing 2 μg of plasmid in a 2 mm electroporation cuvette. H9c2 cells were transfected with the electrical parameters of T-20 (Jung et al., 2007).

GST pull-down assay

GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) with isopropyl-β-D-thiogalactopyranoside induction. Subsequently, cells were sonicated in ice-cold lysis buffer (200 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 100 μM EDTA, 0.1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride). GST fusion proteins were incubated with glutathione-coated beads for 4 hr at 4°C, and normalized for the protein concentration. pCNS/LDHB was translated *in vitro* with TNT® Quick Coupled Transcription/Translation Systems (Promega Corp., Madison, WI). 2 μg of pCNS/LDHB was incubated with 10 μCi of ^{35}S-methionine in TNT® Quick Master mix for 90 min at 30°C. *In vitro*-translated products were incubated mixed with either GST or GST-NHE1.cd fused proteins in E1A binding buffer (50 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol) for 4 hr at 4°C. After washing three times in E1A binding buffer, samples were treated with SDS-sample buffer. Subsequently, samples were subjected to SDS-PAGE, and dried gel was exposed to X-ray film.

Immunoprecipitation and Western Analysis

H9c2 cells were harvested, lysed in mammalian lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.4 mM PMSF), sonicated, and centrifuged at 13,000 rpm for 10 min. For co-immunoprecipitation assay, cell lysates were incubated for 2 hr at 4°C with anti-LDHB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Then protein A/G-agarose beads (Santa Cruz Biotechnology) were added and incubated for 2 hr at 4°C. Immuno-precipitated beads were washed with mammalian lysis buffer. Immunoprecipitates and whole cell lysates (WCLs) were subjected to SDS-PAGE, separated, transferred to a nitrocellulose membrane and immunoblotted with anti-LDHB and anti-NHE1 antibody (Sardet et al., 1990).

Intracellular pH (pH_i) Measurement

Na^+/H^+ exchange activity was measured using a standard protocol with some modifications (Han et al., 2006; Kim et al., 2007). The intracellular pH (pH_i) was measured by pH-sensitive fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM). Transfected cells grown on glass coverslips were washed with PBS, were incubated for 20 min at 37°C in a 5% CO_2 incubator with BCECF in HBSS. BCECF fluorescence was recorded at excitation wavelengths of 440 and 490 nm using the recording setup (Delta Ram, PTI Inc., Brunswick, NJ).

Caspase 3 activation assay

Forty-eighth after transfection, cells were harvested and resuspened in ice-cold lysis buffer (25 mM Hepes, pH 7.4, 0.25% Nonidet P-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 mM EDTA, 2 mM dithiothreitol and 10 mM digitonin). The lysates were clarified by centrifugation and the supernatants were used for caspase assays. Reaction buffer containing 10 mM DTT and substrate 2.5 mM DEVD-AMC (Peptron, Korea) were added to each sample and carried out at 37°C for 30 min. The detection of caspase 3 activity was measured using Luminescence Spectrometer LS50B (Perkin-Elmer, UK) at excitation and emission wavelengths, 360 and 460 nm.

Measurement of Cell Death

The 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining protocol was modified slightly, as described

previously (Pollenz et al., 1992). Cells were grown on coverslips in 6-well culture plates. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, washed with PBS, stained with 2 mg/mL of DAPI, and counted under a fluorescent microscope (Zeiss Axiovert, Zeiss, Germany).

RESULTS AND DISCUSSION

Yeast two-hybrid analyses isolated LDHB as a novel binding partner of NHE1

Yeast two-hybrid system was performed to identify candidate NHE1.cd partners from HeLa cDNA library. NHE1.cd fused to a pLexA binding domain was used as bait. HeLa cDNA library fused to the B42 activation domain (AD) was utilized as target. Positive clones were selected based on the expressions of β-galactosidase reporter and *leu* genes, which allowed growth on leucine-deficient plates. Among positive clones, clone 9 was selected (Fig. 1A, lane 1). The library plasmid isolated from clone 9 was amplified and analyzed by agarose gel electrophoresis after restriction digestion (Fig. 1B). Plasmid insert from clone 9 was sequenced and the sequence was analyzed by a BLAST search. Clone 9 contained insert of 1133 nucleotides containing amino acid residues 25-335 and 200 nucleotide of 3'-untranslated region of the human lactate dehydrogenase B (GenBank accession number NM 002300) (Fig. 2).

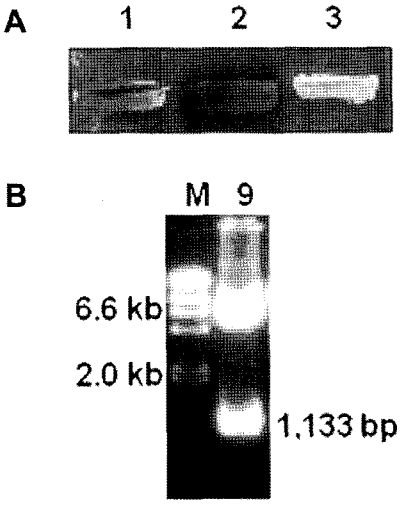

Fig. 1. Yeast two-hybrid system to screen for potential partners of NHE1.cd. *A*, The cotransformants of EGY48 with pLexA/NHE1.cd and clone 9 (pJG4-5/LDHB) (lane 1), pLexA/FADD and pJG4-5/TRADD as a positive control (lane 2) and pLexA/FADD and pJG4-5/Daxx as a negative control (lane 3) were streaked on SD medium containing X-gal as substrate for β-galactosidase. *B*, Clone 9 was rescued from the yeast colony and transformed into *E.coli*. Clone 9 plasmid DNA was purified and the size of insert DNA was analyzed on the 1 % agarose gel after digestion with *Eco*RI and *Xho*I. Size marker was indicated as M (λ*Hind*III).

LDHB interacts with NHE1 *in vitro* and in H9c2 cells

pCNS/LDHB was obtained from the 21C Frontler Human Gene Bank. We examined whether LDHB interacted with NHE1.cd *in vitro*. ^{35}S-labelled LDHB was produced by *in vitro* translation and was incubated with purified glutathione S-transferase (GST) or GST-NHE1.cd immobilized on GST-agarose beads. LDHB interacted with the GST-NHE1.cd fusion protein, but not GST alone (Fig. 3A). Endogenous interaction between LDHB and NHE1 was also examined in H9c2 cells. Anti-LDHB antibody

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                                                CTTGCAGAGCCGGCGCCGGAGGAGACGCAC   30
GCAGCTGACTTTGTCTTCTCCGCACGACTGTTACAGAGGTCTCCAGAGCCTTCTCTCTCCTGTGCAAA   98
ATG GCA ACT CTT AAG GAA AAA CTC ATT GCA CCA GTT GCG GAA GAA GAG GCA ACA GTT CCA  158
AAC AAT AAG ATC ACT GTA GTG GGT GTT GGA CAA GTT GGT ATG GCG TGT GCT ATC AGC ATT  218
CTG GGA AAG TCT CTG GCT GAT GAA CTT GCT CTT GTG GAT GTT TTG GAA GAT AAG CTT AAA  278
GGA GAA ATG ATG GAT CTG CAG CAT GGG AGC TTA TTT CTT CAG ACA CCT AAA ATT GTG GCA  338
GAT AAA GAT TAT TCT GTG ACC GCC AAT TCT AAG ATT GTA GTG GTA ACT GCA GGA GTC CGT  398
CAG CAA GAA GGG GAG AGT CGG CTC AAT CTG GTG CAG AGA AAT GTT AAT GTC TTC AAA TTC  458
ATT ATT CCT CAG ATC GTC AAG TAC AGT CCT GAT TGC ATC ATA ATT GTG GTT TCC AAC CCA  519
GTG GAC ATT CTT ACG TAT GTT ACC TGG AAA CTA AGT GGA TTA CCC AAA CAC CGC GTG ATT  579
GGA AGT GGA TGT AAT CTG GAT TCT GCT AGA TTT CGC TAC CTT ATG GCT GAA AAA CTT GGC  638
ATT CAT CCC AGC AGC TGC CAT GGA TGG ATT TTG GGG GAA CAT GGC GAC TCA AGT GTG GCT  698
GTG TGG AGT GGT GTG AAT GTG GCA GGT GTT TCT CTC CAG GAA TTG AAT CCA GAA ATG GGA  758
ACT GAC AAT GAT AGT GAA AAT TGG AAG GAA GTG CAT AAG ATG GTG GTT GAA AGT GCC TAT  818
GAA GTC ATC AAG CTA AAA GGA TAT ACC AAC TGG GCT ATT GGA TTA AGT GTG GCT GAT CTT  878
ATT GAA TCC ATG TTG AAA AAT CTA TCC AGG ATT CAT CCC GTG TCA ACA ATG GTA AAG GGG  938
ATG TAT GGC ATT GAG AAT GAA GTC TTC CTG AGC CTT CCA TGT ATC CTC AAT GCC CGG GGA  998
TTA ACC AGC GTT ATC AAC CAG AAG CTA AAG GAT GAT GAG GTT GCT CAG CTC AAG AAA AGT 1058
GCA GAT ACC CTG TGG GAC ATC CAG AAG GAC CTA AAA GAC CTG TGA CTAGTGAGCTCTAGG    1118
CTGTAGAAATTTAAAAACTACAATGTGATTAACTCGAGCCTTTAGTTTTCATCCATGTACATGGATCAC      1187
AGTTTGCTTTGATCTTCTTCAATATGTGAATTTGGGCTCACAGAATCAAAGCCTATGCTTGGTTTAATG      1256
CTTGCAATCTGAGCTCTTGAACAAATAAAATTAACTATTGTAGTGTGAAAAAAAAAAAAAAAAAA         1321
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Fig. 2. The nucleotide sequence of LDHB. The start codon ATG and the stop codon TGA are marked in bold. The underline marks the insert harbored in the clone 9. GenBank accession number: NM 002300 for hLDHB.

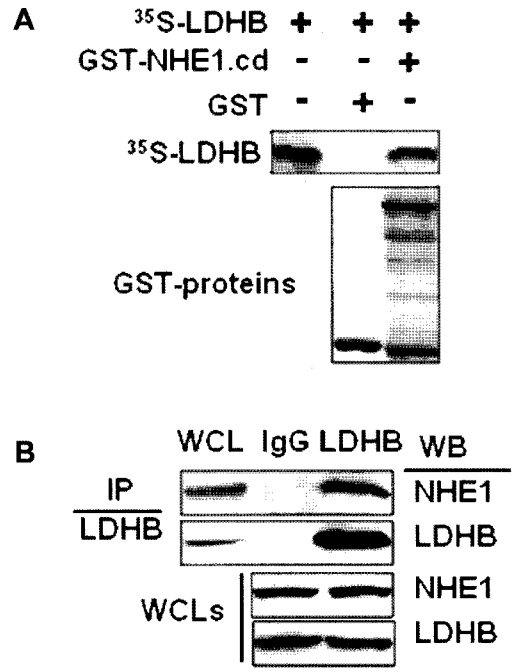

Fig. 3. NHE1 interacts with LDHB. *A*, *In vitro*-translated ^{35}S-labeled LDHB was incubated with GST, GST-NHE1cd immobilized on glutathione-Sepharose beads for 4 hr. ^{35}S-labeled bound proteins were separated on an SDS-PAGE and analysed by autoradiography. Coomassie Brilliant Blue-stained GST fusion proteins were aligned to estimate protein levels. *B*, Endogenous LDHB was immunoprecipitated with anti-LDHB or anti-mouse IgG1 from H9c2 cell lysates. Immunoprecipitates were immunoblotted with indicated antibodies. Whole cell lysates (WCL) were immunoblotted with anti-NHE1 and anti-LDHB antibodies. *WCL*, whole cell lysate; *IP*, immunoprecipitation.

immunoprecipitated both LDHB and NHE1 from H9c2 cell lysates, implying endogenous interaction (Fig. 3B). Our results clearly show that NHE1 associates with LDHB.

LDHB activates NHE1 transporter

We overexpressed LDHB in H9c2 cells, and measured NHE1 activity by determining the intracellular pH (pH_i). NHE1 restores the pH_i by extruding protons upon acidosis induced by stress, and alkalinizes cells (Hooley et al., 1996). The intracellular pH recovery in the LDHB-transfected cells was more rapid than that in alkalinization cells, indicating that LDHB stimulates NHE1 to extrude H^+ (Fig. 4). The steady-state pH_i value in control cells was 7.4 ±0.19. However, intracellular alkalization was induced in LDHB-transfected cells (8.17±0.23). Therefore, our data show that LDHB stimulates NHE1 transporter activity.

The C-terminal region of NHE1 interacts with various proteins, including p38, PP1, PP2A, 14-3-3, HSP70, Bin1, Daxx, tescalcin, Ca^{2+}/calmodulin, CHP family, PIP_2, ezrin, radixin, and moesin (Lehoux et al., 2001; Mailänder et al., 2001; Xue et al., 2007; Zaun et al., 2008). Some interacters regulate the transporter activity of NHE1. The PP1-NHE1 interaction dephosphorylates NHE1 and inhibits activation

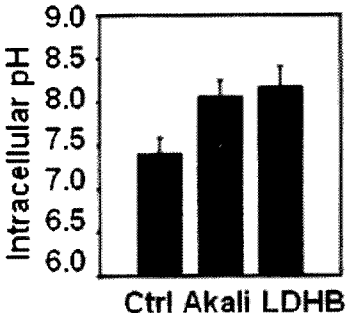

Fig. 4. LDHB activates NHE1. H9c2 cells were transfected with 2 μg of indicated plasmids. At 48 hr after transfection, intracellular pH (pHi) was measured using BCECF in a thermostated stirred cuvette in a fluorescence spectrophotometer as described in Materials and Methods. H^+ efflux rates were calculated at various pH_i. *Ctrl*, cell only; *Akali*, alkalinization.

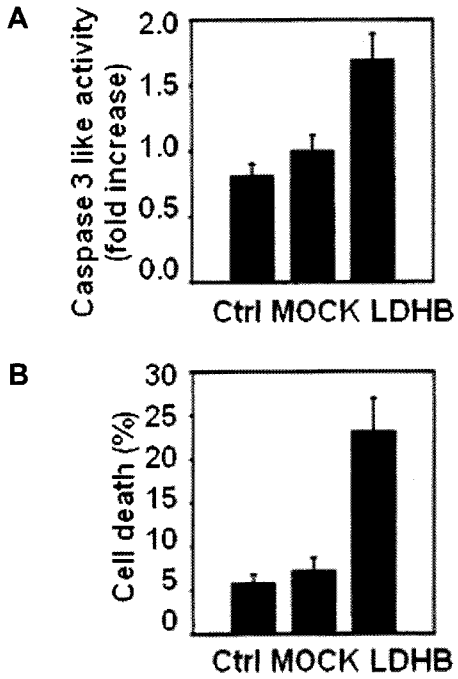

Fig. 5. LDHB promotes cell death. *A*, H9c2 cells were transfected with 2 μg of plasmids. At 48 hr after transfection, *in vitro* caspase 3 activity was measured by relative fluorescence intensity using a spectrofluorometer. The data are mean±SEM (n=3). *B*, H9c2 cells were transfected with 2 μg of plasmids. At 48 hr after transfection, cells were fixed and stained with DAPI. Nuclear condensation was determined by counting at least 300 cells. The data in the bar graph are mean±SEM (n=3). *Ctrl*, cell only.

of NHE1. p38 phosphorylates NHE1 upon interaction and activates NHE1. Daxx also binds to NHE1 and enhances its transporter activity. Therefore, LDHB enlists itself to the list of positive regulators of NHE1 such as p38 and Daxx.

LDHB induces cell death

The effect of LDHB overexpression upon caspase activation in H9c2 cells was examined. After transfection with vector and pCNS/LDHB, the caspase 3 like activity in cell extracts

was measured by the cleavage of the fluorescent peptide DEVD-AMC. The cleavage of substrate was increased significantly in cells with transfected LDHB when compared to those with vector control (Fig. 5A). Our data clearly demonstrate that overexpression of LDHB activates caspase 3.

The nuclear condensation induced by LDHB was examined by DAPI staining after transfection with vector or pCNS/LDHB. Transient transfection of the pCNS/LDHB efficiently induced cell death in H9c2 cells (23.1± 3.8%) when compared with that in vector-transfected H9c2 cells (7.2±1.5%; Fig. 5B). Our data clearly demonstrate that transfection of LDHB causes a significant cell death.

Typical apoptotic phemomena are such as caspase activiation, phosphatidyl serine traslocation in the cell membrane, membrane blebbing, and chromatin condensation (Okada and Mak, 2004). The cell death induced by LDHB overexpression showed typical apoptotic features, chromatin condensation and activation of caspase 3.

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