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SAP97 promotes the stability of Na_x channels at the plasma membrane

Masahito Matsumoto [a], Akihiro Fujikawa [a], Ryoko Suzuki [a], Hidetada Shimizu [a], Kazuya Kuboyama [a], Takeshi Y. Hiyama [a,b], Randy A. Hall [c], Masaharu Noda [a,b,*]

[a] Division of Molecular Neurobiology, National Institute for Basic Biology, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan
[b] School of Life Science, The Graduate University for Advanced Studies, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan
[c] Department of Pharmacology, Rollins Research Center, Emory University School of Medicine, Atlanta, GA 30322, USA

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ABSTRACT

Na_x is a sodium-level sensor for body fluids expressed in the circumventricular organs in the brain. Na_x has a putative PSD95/Disc-large/ZO1 (PDZ)-binding motif at the carboxyl (C)-terminus. Here we found that several PDZ proteins bind to Na_x by PDZ-array overlay assay. Among them, synapse-associated protein 97 (SAP97/DLG1) was coexpressed with Na_x in the subfornical organ. In C6 glioblastoma cells, destruction of the PDZ-binding motif of Na_x or depletion of SAP97 resulted in a decrease in cell-surface Na_x, which was attenuated with inhibitors of endocytosis. These results indicate that SAP97 contributes to the stabilization of Na_x channels at the plasma membrane.

Structured summary of protein interactions:
Nax physically interacts with **SAP97** by anti tag coimmunoprecipitation (View interaction)
CNRasGEF binds to **Nax** by protein array (View interaction)
Nax and **SAP97** colocalize by fluorescence microscopy (View interaction)
GIPC1 binds to **Nax** by protein array (View interaction)
ZO-1 binds to **Nax** by protein array (View interaction)
SAP97 binds to **Nax** by protein array (View interaction)
Densin-180 binds to **Nax** by protein array (View interaction)
Beta-1-syntrophin binds to **Nax** by protein array (View interaction)
ERBIN binds to **Nax** by protein array (View interaction)
Nax physically interacts with **SAP97** by pull down (View interaction)
Lnx1 binds to **Nax** by protein array (View interaction)
nNOS binds to **Nax** by protein array (View interaction)

1. Introduction

Sodium (Na) is a major electrolyte of extracellular fluids and the main determinant of osmolality. Na homeostasis is essential to life and Na^+ concentrations in plasma and cerebrospinal fluid (CSF) are continuously monitored to maintain a physiological level of Na^+ in body fluids [1]. We have previously shown that Na_x, which structurally resembles voltage-gated sodium channels ($Na_v1.1$–1.9), is a concentration-sensitive Na channel with a threshold of ~150 mM for extracellular Na^+ concentration $[Na^+]_o$ in vitro [2–4].

In the brain, Na_x channels are specifically expressed in astrocytes and ependymal cells in the sensory circumventricular organs (sCVOs), such as the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), where Na_x-positive glial cells are involved in sensing an increase in $[Na^+]$ in body xfluids [5]. *Na_x*-Deficient mice do not stop ingesting salt even when dehydrated, while wild-type mice avoid salt [6]. This behavioral defect of *Na_x*-deficient mice is recovered by a site-directed transfer of the *Na_x* gene with an adenoviral vector into the SFO [7]. Na_x thus functions as the brain's Na^+-level sensor for the homeostatic control of $[Na^+]$ in body fluids.

Some PDZ domain-containing proteins are known to serve as key scaffolds for channel proteins to control their trafficking [8]. PDZ domains are 90 amino-acid protein–protein interaction modules that bind to specific C-terminal motifs in their target proteins [9]. Most of the target proteins have a conserved PDZ-binding

* Corresponding author at: Division of Molecular Neurobiology, National Institute for Basic Biology, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan. Fax: +81 564 59 5845.
E-mail address: madon@nibb.ac.jp (M. Noda).

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motif that matches one of the three 'canonical' consensus motifs (Class I, –X–S/T–X–L/V; Class II, –X–Ψ–X–Ψ; Class III, –X–D/E–X–L/V; Ψ indicates hydrophobic amino acid; [9]). Giallourakis et al. [9] recently found a new consensus motif (–X–S/T–X–I/A) other than the 'canonical' motifs. Because the C-terminal sequence of Na_x (–Q–T–Q–I for rat and mouse, and –Q–S–Q–I for human) fits this 'non-canonical' PDZ-binding motif, we hypothesized that the Na_x channel may be regulated by PDZ-scaffold proteins. In the present study, we took advantage of a proteomic PDZ-domain array [10], containing 96 distinct PDZ domains, to screen for PDZ proteins that might interact with Na_x. Among the PDZ proteins thus identified, we found that SAP97 (also known as DLG1) is coexpressed with Na_x in the SFO, and contributes to the stability of Na_x channels in the plasma membrane.

2. Materials and methods

2.1. Recombinant proteins

The Glutathione S-transferase (GST)-fused protein with the C-terminal region (amino acid residues 1489–1681) of mouse Na_x (GST-Na_x-C-term), its PDZ-binding-motif mutant (GST-Na_x-C-term-T1679A) in which Thr-1679 was replaced with Ala, or its PDZ-binding-motif deletion mutant (GST-Na_x-C-termΔTQI) was expressed in an *Escherichia coli* strain, BL21, and purified by glutathione affinity chromatography as described [11].

2.2. Overlay assay on the PDZ array

The PDZ-array overlay assay was performed as described [10,12]. Briefly, a nylon membrane spotted with a series of His/S-tagged PDZ domain proteins was pre-treated with a blocking buffer containing 2% non-fat dry milk and 0.1% Tween-20 in 150 mM NaCl and 10 mM phosphate buffer, pH 7.3 (PBS) for 30 min, then overlaid with the GST-fused proteins (15 nM) in the

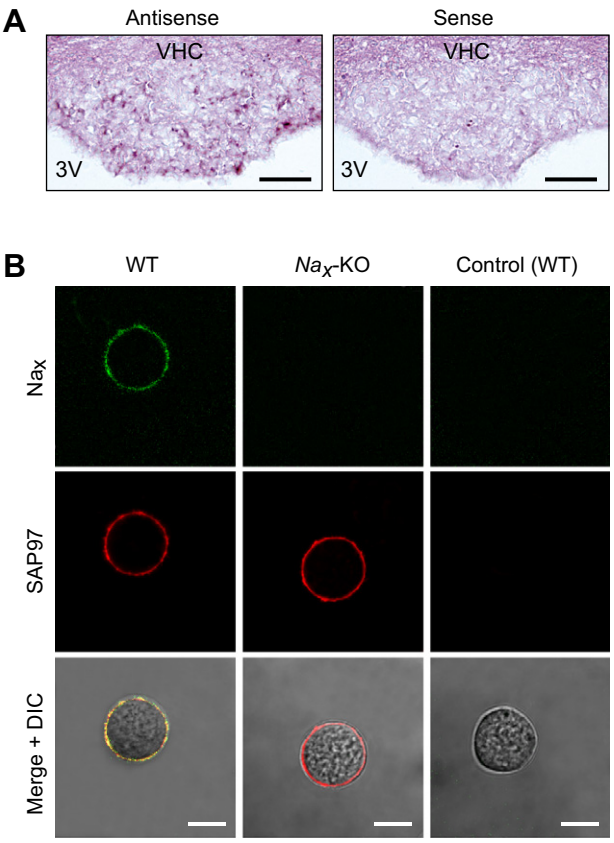

Fig. 2. Expression of Na_x and SAP97. (A) Expression of *SAP97* in the SFO. Coronal tissue sections of the mouse SFO were hybridized with antisense SAP97, or with the control sense probe. VHC, ventral hippocampal commissure; 3V, dorsal third ventricle. Scale bars, 50 μm. (B) Double immunostaining of SFO cells obtained from wild type (WT; left) and *Na_x*-knockout (*Na_x*-KO; middle) mice with anti-Na_x and anti-SAP97 antibodies. Right panels show the results of control experiments omitting the primary antibodies. Scale bars, 10 μm.

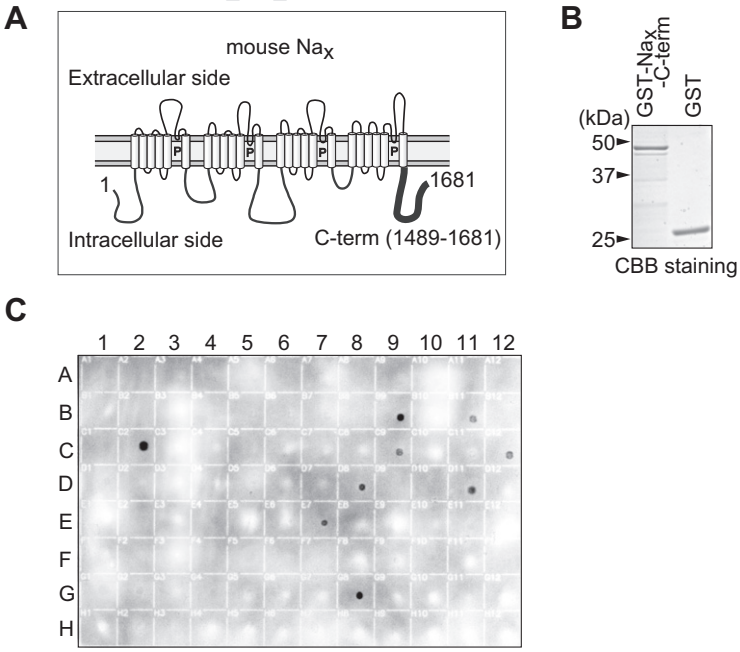

Fig. 1. Screening of PDZ proteins that bind to the C-terminus of Na_x. (A) Schematic drawing of Na_x. The C-terminal region of Na_x used for preparation of the recombinant GST-fused protein (GST-Na_x-C-term) is indicated with a bold line. (B) Preparation of GST-Na_x-C-term and control GST proteins. The purity was checked by Coomassie Brilliant Blue R-250 (CBB) staining following SDS–PAGE. (C) PDZ array overlay assay with GST-Na_x-C-term. For the proteins spotted on the array, see Supplementary Information (Table S1). Strong positive hits were observed for CNRasGEF-PDZ (B9), SAP97-PDZ1+2 (C2), and LNX1-PDZ3 (G8).

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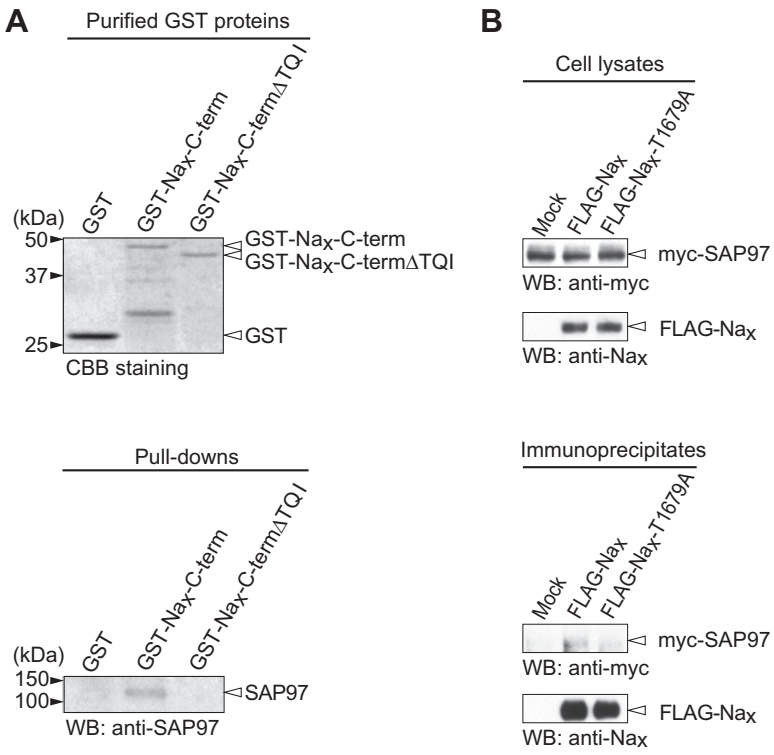

Fig. 3. Binding of SAP97 with Na_x. (A) In vitro binding assay of SAP97 and the C-terminal region of the Na_x channel. Input proteins were visualized by CBB staining (upper). Glutathione beads coated with GST, GST-Na_x-C-term, or GST-Na_x-C-termΔTQI (PDZ-binding-motif deletion mutant) were incubated with the lysate of C6 cells. The bound endogenous SAP97 was detected by Western blotting with anti-SAP97 (lower). (B) Binding of the full-length Na_x to SAP97 in HEK293T cells. The expression construct for myc-tagged SAP97 was transfected into HEK293T cells, together with the control vector, FLAG-tagged wild-type Na_x, or its FLAG-tagged PDZ-binding-motif mutant. The amounts of protein expressed in the cell extract (upper two panels), and immunoprecipitated with anti-FLAG M2 (lower two panels), were analyzed by Western blotting using anti-myc (for detection of SAP97) and anti-Na_x.

blocking buffer. The binding was detected with a horseradish peroxidase-conjugated anti-GST antibody (GE Healthcare), and visualized by enhanced chemiluminescence (GE Healthcare).

2.3. In situ hybridization

In situ hybridization was performed as described [13] on coronal brain sections of C57BL/6 mice (male, 4 months old) by using cRNA probes synthesized from pGEM T-Easy vector (Promega) carrying the 812-base fragment of mouse SAP97 (nucleotides 694–1506, GenBank accession number NM_007862).

2.4. Dissociation of the SFO cells

The SFO was dissected from fresh brain slices of *Na_x*-knockout [6] or control C57BL/6 mice (male, 2 months old), and SFO cells were dissociated as described [2]. Isolated cells were attached on glass-bottomed dishes coated with Cell-tak (BD Biosciences) in DMEM containing 10% fetal calf serum (FCS) in a humidity-controlled incubator gassed with 5% CO_2 for 3 h at 37 °C.

2.5. Immunofluorescence cell staining

SFO cells and rat C6 cells were fixed with 4% paraformaldehyde in PBS, and microwaved in 10 mM citrate buffer, pH 6.0. After blocking with 4% non-fat dry milk and 0.1% Tween 20 in PBS, the cells were incubated with rabbit anti-Na_x [2] and mouse anti-SAP97 (Cat. No. 610874; BD Biosciences) in the blocking buffer for 1 week at 4 °C. Bound antibodies were visualized with Alexa488-conjugated anti-rabbit and Alexa594-conjugated anti-mouse antibodies (Life Technologies), respectively.

2.6. Expression plasmid construction and DNA transfection to HEK293T cells

The expression construct of the PDZ-binding motif mutant (pTRE-mNa_x-T1679A), in which Thr-1679 was replaced with Ala, was generated from pTRE-mNa_x [11] using the QuickChange Mutagenesis kit (Stratagene). Expression plasmids for the N-terminal FLAG-tagged wild-type Na_x (pFLAG-mNa_x) and mutant Na_x (pFLAG-mNa_x-T1679A) were generated by subcloning the respective cDNAs, into pcDNA-FLAG [14]. The construct for myc-tagged SAP97 (pMyc-SAP97) was prepared as follows. The full-length cDNA of mouse SAP97 (GenBank accession number NM_007862) was obtained by RT-PCR with mouse brain mRNA, and a myc-epitope tag was attached to the N-terminus of the cDNA using PCR. Then it was inserted into a mammalian expression vector pcDNA3.1 (Life Technologies). HEK293T cells (human embryonic kidney epithelial cells) were maintained in DMEM containing 10% FCS under 5% CO_2 at 37 °C. DNA transfection was performed by the standard calcium phosphate method.

2.7. Na_x-expressing C6 cells

The C6M16 cell line, in which mouse Na_x expression is inducible under the control of the tetracycline-responsive element (TRE), was described previously [11]. Cell lines which allow inducible expression of a PDZ-binding-motif mutant of Na_x were established in the same way. Briefly, C6 glioblastoma cells were cotransfected

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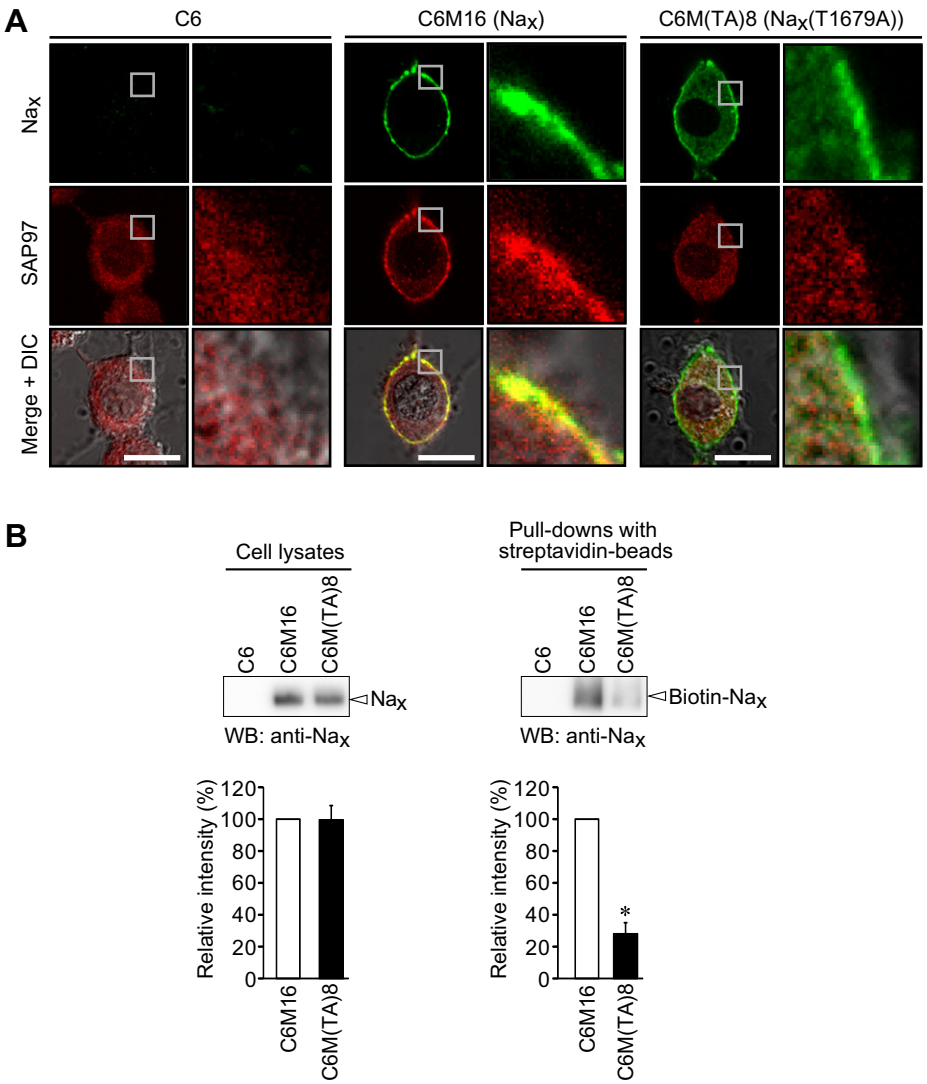

Fig. 4. Decrease in cell-surface expression of the Na_x mutant with Thr-1679 changed to Ala in C6 cells. (A) C6 cells (without expression of Na_x), C6M16 cells (with expression of wild-type Na_x) or C6M(TA)8 cells (with expression of mutant Na_x in the PDZ-binding motif) were fixed, permeabilized, and then stained with anti-Na_x (green) and anti-SAP97 (red). The fluorescence images merged with differential interference contrast images (DIC) are shown at the bottom. The right images are enlargements of the area enclosed by the square in the left adjacent image, respectively. Scale bars, 10 μm. (B) Western blots of the total cell lysate (left) and biotinylated cell-surface proteins (right) from C6, C6M16, and C6M(TA)8 cells using the anti-Na_x. Signal intensities were quantified by densitometry and shown as a percentage of the intensity of C6M16. Data are the mean ± SE, $n = 4$ for each; $^*P < 0.01$, two-tailed t test.

with pTRE-mNa_x-T1679A and pKJ2 carrying the neomycin-resistance gene, and then selected with a neomycin-analog G418 (1.2 mg/ml) in DMEM containing 10% FCS under 5% CO_2 at 37 °C. Na_x expression was induced by the Tet-Off adenoviral vector (Clontech) in serum-free DMEM containing 1 mM dibutyryl cyclic AMP for 36 h. Expression of Na_x was estimated by Western blotting. A clone named C6M(TA)8, which shows the same expression level as C6M16 cells, was selected and used for the present study.

2.8. Co-immunoprecipitation and GST pull-down assays

Cells were lysed with a lysis buffer (1% Triton X-100 and 150 mM NaCl in 10 mM Tris–HCl, pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Applied Science), and the supernatant was collected by centrifugation. Cell extracts obtained were incubated with anti-FLAG M2 antibody (Sigma–Aldrich), and the immunocomplexes were precipitated using protein G-Sepharose (GE Healthcare). For the GST-pull down assay, GST-Na_x-C-term or GST-Na_x-C-termΔTQI was first bound to glutathione-Sepharose beads (GE Healthcare), and the beads were incubated with C6 cell extracts. After washing the beads, the bound proteins were analyzed by Western blotting.

2.9. Biotinylation of cell-surface proteins

Cells were washed with 10 mM triethanolamine, pH 7.5 containing 150 mM NaCl and 2 mM $CaCl_2$, and treated with 500 μg/ml Sulfo-NHS-Biotin (Pierce) for 30 min on ice. The reaction was quenched by washing with a buffer containing 0.1 M glycine, and then cells were lysed with the lysis buffer. Na_x was immunoprecipitated from the cell extracts with anti-Na_x by using Protein G-coated magnetic beads (Dynabeads protein G, Life Technologies), and subjected to Western blotting as above.

2.10. RNA interference

Predesigned siRNA against rat SAP97 (siRNA ID, SASI_Rn02_00260207) and its scrambled siRNA were purchased from

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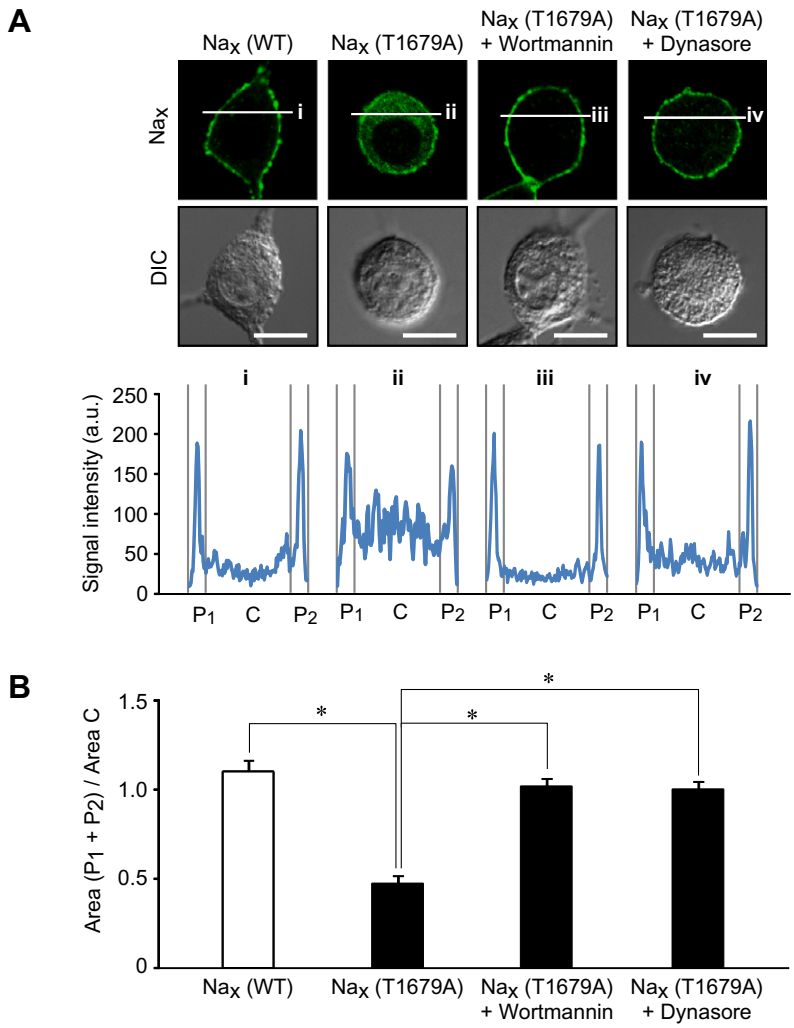

Fig. 5. Improvement in surface expression of the Na_x(T1679A) mutant in C6 cells on treatment with wortmannin and dynasore. (A) Upper panels: Subcellular distribution of the wild-type and T1679A mutant Na_x in C6 cells. After the induction of Na_x(T1679A) channels, cells were treated with 100 nM wortmannin or 200 μM dynasore for 6 h. Then the cells were fixed, permeabilized, and stained with anti-Na_x. Scale bars, 10 μm. Lower graphs: Fluorescence intensity profiles along the white lines in the upper panels. The profile was divided into three parts (P_1:C:P_2 = 15:70:15 in length). a.u., arbitrary unit. (B) Subcellular distribution of Na_x. The relative fluorescence intensity of the membrane region to the central region in (A) is presented respectively as the mean ± SE (n = 8 for each); $^*P < 0.01$, ANOVA followed by Scheffe's test.

Sigma–Aldrich. C6 cells (2×10^6) were electroporated with 100 pmol siRNA using the Amaxa Nucleofector (Amaxa) according to the manufacturer's protocol.

2.11. Intracellular Na^+ imaging

Intracellular Na^+ imaging with sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM; Molecular Probes) was performed as described [2].

3. Results

3.1. Identification of PDZ proteins that interact with Na_x

We screened for potential interactions between Na_x and PDZ proteins using the PDZ-array overlay assay [10,12] with the GST-fused protein with the C-terminal region of Na_x (GST-Na_x-C-term) (Fig. 1). Proteins spotted on the array are shown in Supplementary Information (Table. S1). Strong positive hits on the first and second PDZ domains (PDZ1+2) of SAP97, the PDZ domain of CNRasGEF (also called PDZ-GEF1, RA-GEF-1, or RAPGEF2), and the third PDZ domain (PDZ3) of LNX1 were identified, along with weaker positive hits on neuronal nitric oxide synthase (nNOS), Erbb2 interacting protein (ERBIN also called LAP2), PDZ3 of zonula occludens-1 (ZO1, also called Tjp1), GAIP-interacting protein C-terminus (GIPC, also called synectin or TIP-2), β1-syntrophin, and DENSIN180 (also called Lrrc7). No binding was detected with GST-Na_x-C-term-T1679A (Fig. S1) or control GST (data not shown), indicating that the native C-terminal PDZ-binding sequence of Na_x is essential for the binding with all the potential partners.

3.2. Coexpression of SAP97 and Na_x in SFO cells

Among the three strongly positive PDZ proteins, SAP97 was found to be expressed in the SFO by in situ hybridization (Fig. 2A), while CNRasGEF and LNX1 were not (data not shown). Moreover, immunostaining of mouse SFO cells with anti-SAP97 and anti-Na_x antibodies revealed robust colocalization of the two proteins at the plasma membrane (Fig. 2B).

3.3. Interaction of Na_x with SAP97 via its C-terminal PDZ-binding motif

GST-Na_x-C-term, but not GST-Na_x-C-termΔTQI (PDZ-binding-motif deletion mutant), pulled down the native SAP97 from the C6

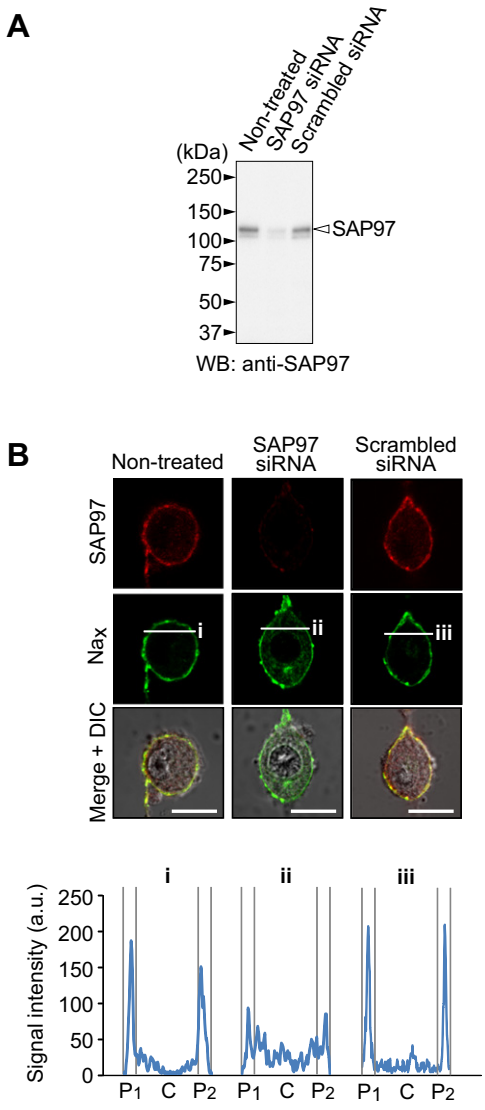

Fig. 6. Reduction in the cell-surface expression of Na_x with depletion of SAP97. (A) Depletion of SAP97 in C6M16 cells. Expression of SAP97 in non-treated C6M16 cells, or C6M16 cells transfected with siRNA for SAP97 or scrambled siRNA was examined by Western blotting. (B) Subcellular distribution of Na_x. The same cells as above were immunostained with anti-SAP97 and anti-Na_x. The fluorescence intensity profiles along the white lines are shown below. Scale bars, 10 μm. a.u., arbitrary unit.

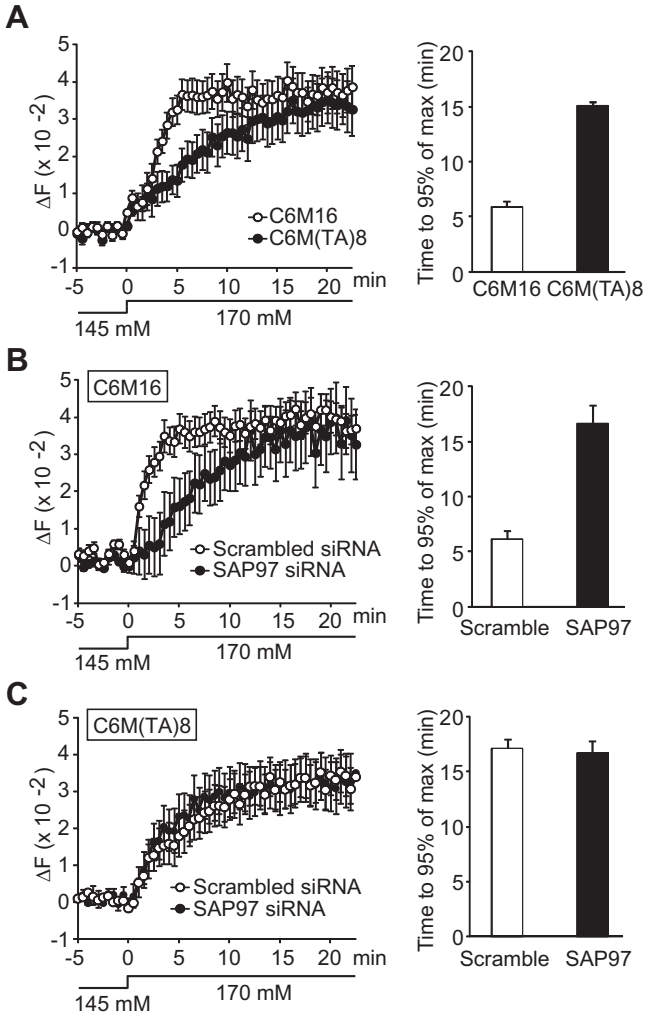

Fig. 7. Reduced sodium influx in C6 cells in the absence of any association between Na_x and SAP97. (A) Left: Na^+ imaging of C6M16 (wild-type Na_x) and C6 M(TA)8 (T1679A mutant Na_x) cells upon elevation of the extracellular Na^+ concentration from 145 mM to 170 mM. The coordinate gives the fluorescence ratio (ΔF340/380 nm) of SBFI, representing the intracellular Na^+ concentration. The physiological 145 mM Na^+ solution was changed to a 170 mM solution at 0 min. Right: Summary of the time to reach 95% of the maximum fluorescence ratio. (B, C) Left: Na^+ imaging of C6M16 (B) or C6 M(TA)8 (C) cells expressing siRNA for SAP97 or the control scrambled siRNA. Right: Summary of the time to reach 95% of the maximum fluorescence ratio. Data represent the mean ± SE ($n = 40$ for each); $^*P < 0.01$, two-tailed t test.

cell lysate (Fig. 3A). We next examined the binding of the full-length Na_x channel and SAP97 in a cellular context. HEK293T cells were transfected with myc-tagged SAP97, together with FLAG-tagged Na_x, FLAG-tagged Na_x(T1679A), or a control mock vector (pcDNA-FLAG). When the cell extracts were immunoprecipitated with anti-FLAG antibody, myc-tagged-SAP97 was coimmunoprecipitated with FLAG-tagged Na_x, but not with the FLAG-tagged T1679A mutant or the control (Fig. 3B). These results verified that Na_x binds to SAP97 through its C-terminal PDZ-binding motif in cells.

3.4. Decreased surface expression of the PDZ-binding-motif mutant of Na_x

We previously established a C6 glioblastoma cell line, C6M16, in which the expression of Na_x channels is inducible under the control of the TRE [11]. For the present study, we established another C6 cell line, C6M(TA)8 expressing the Na_x(T1679A) mutant with a similar expression level of Na_x proteins to C6M16 cells, to characterize differences from the wild type.

In the parent C6 cells without expression of Na_x, SAP97 was detected in the intracellular region (Fig. 4A, left panels). In contrast, in the C6M16 cells with Na_x expression, SAP97 was clearly observed at the plasma membrane in addition to the cytoplasm (Fig. 4A, middle panels). However, in C6M(TA)8 cells, the signal intensity for Na_x(T1679A) at the plasma membrane was markedly decreased compared to the wild-type Na_x and strong signals were detected in the cytoplasm (Fig. 4A, right panels). Indeed, cell-surface biotinylation indicated the surface expression of the mutant Na_x to be significantly lower than that of the wild-type, while the total amount was equivalent (Fig. 4B). These results suggest that translocation of Na_x channels to the plasma membrane was impaired, or translocation from the plasma membrane to cytoplasmic area was enhanced, by the loss of function of the PDZ-binding domain. Moreover, the finding that SAP97 is present at the plasma

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membrane in C6M16 cells, but not in C6M(TA)8 or the parent C6 cells, also indicate that the targeting of SAP97 to the plasma membrane is dependent on the binding with Na_x.

3.5. Improvement of the surface expression of the PDZ-binding-motif mutant of Na_x by inhibition of endocytosis

We postulated that SAP97 contributes to the stabilization of Na_x at the plasma membrane. If this was the case, inhibitors of endocytosis should increase the surface expression of the T1679A mutant of Na_x. Consistent with this idea, incubation with wortmannin, an inhibitor of endocytosis [15], or dynasore, an inhibitor for dynamin-dependent endocytosis [16], markedly ameliorated the surface expression of the Na_x(T1679A) mutant (Fig. 5). On the other hand, the surface expression of wild-type Na_x was not affected with these inhibitors (data not shown). These results indicate that binding with SAP97 via the PDZ-binding motif of Na_x promotes the stabilization of Na_x at the plasma membrane.

3.6. Decrease in the surface expression of Na_x by reduction of SAP97 expression

To examine whether the surface expression of Na_x channels is stabilized by endogenous SAP97, we electroporated siRNA to knockdown SAP97 in C6M16 cells. Along with the decrease in SAP97 expression (Fig. 6A), the surface expression of wild-type Na_x was found to be markedly decreased (Fig. 6B).

3.7. Decrease in Na_x-mediated sodium influx by PDZ-binding-motif mutation or SAP97 knockdown

Finally, we determined the functional relevance of the binding of Na_x with SAP97 by performing Na^+-imaging studies. When the extracellular Na^+ concentration, $[Na^+]_o$ was increased from 145 mM to 170 mM, both C6M16 cells expressing wild-type Na_x and C6M(TA)8 cells expressing the Na_x(T1679A) mutant showed increases in the intracellular Na^+ concentration, $[Na^+]_i$, and the level eventually reached the same equilibrium point between Na^+ influx by Na_x and Na^+ export by Na^+/K^+-ATPase (Fig. 7A). However, importantly, C6M(TA)8 cells took longer to reach this equilibrium level than control C6M16 cells. This indicates that the reduction in the number of surface Na_x channels diminished the Na^+ influx.

Similarly, when SAP97 expression in C6M16 cells was knocked down with siRNA, the time taken to reach the plateau of $[Na^+]_i$ also increased to approximately the same as that observed in C6M(TA)8 cells (Fig. 7B). Importantly, knockdown of SAP97 in C6M(TA)8 cells did not slow down the rate of increase in $[Na^+]_i$ (Fig. 7C). These findings clearly indicate that the cell-surface expression of Na_x channels is proportional to the increase in $[Na^+]_i$ in response to the increase in $[Na^+]_o$.

4. Discussion

In this study, we found that the PDZ-binding motif at the C-terminus of Na_x associates with SAP97 in cells. SAP97 was coexpressed with Na_x in glial cells in the SFO, where Na_x monitors the Na^+-level in body fluids. Disruption of the PDZ-binding motif or depletion of SAP97 attenuated Na_x-mediated sodium influx in glial cells due to the impaired cell-surface expression of Na_x. SAP97 appears to ameliorate the cell-surface expression of Na_x channels by enhancing protein stability.

Among voltage-gated sodium channels, $Na_v1.5$ has also been reported to interact with SAP97 via its C-terminal PDZ-binding motif, and the amount of $Na_v1.5$ on the cell surface is decreased in SAP97-silenced myocytes, although the mechanism remains unclear [17]. SAP97 is a member of the membrane-associated guanylate kinase (MAGUK) family along with PSD-95, PSD-93, and SAP102. MAGUK family members are known to regulate the function, localization, and trafficking of ion channels and receptors [8,18–20]. We found that both the mutation of the PDZ-binding motif and depletion of SAP97 attenuated the sodium influx in response to the increase of $[Na^+]_o$, due to the decrease in the amount of Na_x at the cell surface (Fig. 4). Thus SAP97 appears to be an important component for the $[Na^+]_o$-sensing mechanism in the SFO, which is involved in regulation of the surface expression of the sensor channel.

We also identified a number of candidate proteins other than SAP97, which may interact with Na_x (Fig. 1C). CNRasGEF is a guanine nucleotide exchange factor responsible for sustained activation of the Rap1/2 small GTPase [21]. LNX1, an E3 ubiquitin-protein ligase, is expressed in the non-myelinating perisynaptic Schwann cells in adult mice [22]. Although these molecules were not detected in the SFO, further study is required to make clear whether these proteins interact with Na_x in other Na_x-positive cell types, such as non-myelinating Schwann cells, dorsal root ganglion neurons, and Alveolar type II cells [23].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.018.

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