Mutations in the NIPA1 (SPG6) gene, named for "nonimprinted in Prader-Willi/Angelman" has been implicated in one form of autosomal dominant hereditary spastic paraplegia (HSP), a neurodegenerative disorder characterized by progressive lower limb spasticity and weakness. However, the function of NIPA1 is unknown. Here, we show that reduced magnesium concentration enhances expression of NIPA1 suggesting a role in cellular magnesium metabolism. Indeed NIPA1 mediates Mg$^{2+}$ uptake that is electrogenic, voltage-dependent, and saturable with a Michaelis constant of 0.69 ± 0.21 mM when expressed in Xenopus oocytes. Subcellular localization with immunofluorescence showed that endogenous NIPA1 protein associates with early endosomes and the cell surface in a variety of neuronal and epithelial cells. As expected of a magnesium-responsive gene, we find that altered magnesium concentration leads to a redistribution between the endosomal compartment and the plasma membrane; high magnesium results in diminished cell surface NIPA1 whereas low magnesium leads to accumulation in early endosomes and recruitment to the plasma membrane. The mouse NIPA1 mutants, T39R and G100R, corresponding to the respective human mutants showed a loss-of-function when expressed in oocytes and altered trafficking in transfected COS7 cells. We conclude that NIPA1 normally encodes a Mg$^{2+}$ transporter and the loss-of-function of NIPA1 (SPG6) due to abnormal trafficking of the mutated protein provides the basis of the HSP phenotype.

The NIPA1 [NT_078094] gene is named for “nonimprinted in Prader-Willi/Angelman” because it was thought to be located among about 30 imprinted genes linked to chromosome 15q11-q13 (SPG6 locus) involved in the Prader-Willi syndrome (1–5). However, NIPA1 has also been implicated in another distinct disorder termed autosomal dominant hereditary spastic paraplegia (HSP)4 (OMIM 608145 and 600363). HSP comprises more than 30 genetic disorders whose predominant feature is a spastic gait (6). Mutations in at least six genes have been associated with autosomal dominant HSP including NIPA1 (SPG6). This heterogenous group presents with progressive lower limb spasticity and weakness. In the absence of other clinical features these disorders are referred to as pure or uncomplicated HSP (6). Fink et al. (7, 8) reported that uncomplicated HSP was linked to chromosome 15q, the region of NIPA1. Additional studies by this group identified a nucleotide substitution at position 134 of the NIPA1 cDNA that resulted in an amino acid substitution at position 45 of the NIPA1 protein (T45R) in SPG6-linked HSP kindred and in an unrelated kindred that was too small for linkage analysis (9). More recently, three different research groups have identified a missense substitution in NIPA1, G106R, in a number of large unrelated families (10–12). The functional role of NIPA1 in Prader-Willi or HSP syndromes has not been determined.

Magnesium is the second most abundant cation within the cell and plays an important role in many intracellular biochemical functions (13). Despite the abundance and importance of magnesium, little is known about how eukaryotic cells regulate their magnesium content. Intracellular free Mg$^{2+}$ concentration is in the order of 0.5 mM, which is 1–2% of the total cellular magnesium (13). Accordingly, intracellular Mg$^{2+}$ is maintained below the concentration predicted from the transmembrane electrochemical potential. Intracellular Mg$^{2+}$ concentration is finely regulated by precise controls of Mg$^{2+}$ entry, Mg$^{2+}$ efflux, and intracellular storage compartments (14). We have shown that Mg$^{2+}$ entry is through specific and regulated magnesium pathways that are regulated by intrinsic mechanisms so that the culture of cells in media containing low magnesium results in up-regulation of Mg$^{2+}$ uptake into the cells. This adaptive increase in Mg$^{2+}$ entry was shown to be dependent on de novo transcription since prior treatment of the epithelial cells with actinomycin D prevented the adaptation to low extracellular magnesium (15). The data suggest that epithelial cells can sense the environmental magnesium and through transcription- and translation-dependent processes alter Mg$^{2+}$ transport and maintain magnesium balance.

4 The abbreviations used are: HSP, hereditary spastic paraplegia; HA, hemagglutinin; MDCT, mouse distal convoluted tubule; PBS, phosphate-buffered saline; TMD, transmembrane domain.
In an attempt to identify genes underlying cellular changes resulting from adaptation to low extracellular magnesium, we used oligonucleotide microarray analysis to screen for magnesium-regulated transcripts in epithelial cells (16). One transcript, NIPA2, was significantly up-regulated by extracellular magnesium suggesting that the synthesis was regulated by changes in cell magnesium. We showed that NIPA2 mediated Mg\textsuperscript{2+} transport when expressed in Xenopus laevis oocytes. An in silico search demonstrated a second member of this family of proteins, NIPA1. The goal of the present study was to see if NIPA1 mediates Mg\textsuperscript{2+} transport using electrophysiological and fluorescence studies. Furthermore, cellular distribution and subcellular localization was determined by Western blot analysis and immunofluorescence microscopy. Redistribution of NIPA1 protein was evaluated in response to changes in cellular magnesium. Finally, the NIPA1-T39R and G100R mutants, associated with HSP, were created and tested. Our data indicate that NIPA1 protein mediates Mg\textsuperscript{2+} transport and is regulated by magnesium indicating that it may play a role in control of cellular magnesium homeostasis. The T39R and G100R mutations resulted in altered intracellular trafficking of NIPA1 protein and diminished Mg\textsuperscript{2+} transport suggesting a role in the HSP phenotype.

**MATERIALS AND METHODS**

**Construction of Expression Vectors Encoding NIPA1**—A mouse NIPA1 cDNA clone was purchased from RIKEN, catalog number B430207K20. The human NIPA1 cDNA is longer than the mouse sequence due an N-terminal AAAAAA extension. Subclones were sequenced to confirm sequence integrity. The resultant construct, pFLCI-mNIPA1, contained the entire coding region of the mNIPA1 cDNA flanked by 20 bp of untranslated 5’-nucleotide sequence and 929 bp of untranslated 3’-sequence. Two mouse mutant constructs, pFLCI-mNIPA1-T39R and pFLCI-mNIPA1-G100R, in which Thr\textsuperscript{39} (ACG) was replaced with Arg\textsuperscript{39} (AGG) and Gly\textsuperscript{100} (GGG) was replaced with Arg\textsuperscript{100} (AGG), respectively, were produced from pFLCI-mNIPA1 using the QuikChange II XL site-directed mutagenesis kit (Stratagene). A C-terminal HA tag was added to NIPA1 cDNA by PCR with an oligonucleotide (5N1Sac1: 5’-CGAGCTCGGCATGGGGACTG-3’) containing the full HA. NIPA1-HA was then subcloned into the pEGFP-N1 vector replacing the EGFP tag with cRNA, and two-electrode voltage clamp were as described previously and performed at 21 °C (16). Oocytes were studied 3–5 days following injection. Permeability ratios were calculated using the Nernstian relation and apparent \( K_m \) and \( V_{max} \) values with non-linear regression analysis (16).

Epifluorescence microscopy was used to measure Mg\textsuperscript{2+} flux into single oocytes using the Mg\textsuperscript{2+}-responsive mag-fura-2 fluorescence dye (16). Oocytes were injected with 50 \( \mu \)m mag-fura-2 acid (Molecular Probes), 20 min prior to experimentation. The chamber (0.5 ml) was mounted on an inverted Nikon Diaphot-TMD microscope, with a Fluor \( \times \)10 objective, and an I-V association determined. Subsequently, they were clamped at \(-70\) mV for fluorescence measurements for the indicated times. Fluorescence was continuously recorded using a dual-excitation wavelength spectrofluorometer (Delta-scan, Photon Technologies) with excitation for mag-fura-2 at 340 and 385 nm (chopper speed set at 100 Hz) and emission at 505 nm. Results are presented as the 340/385 ratio, which reflects the intracellular Mg\textsuperscript{2+} concentration.

**Animal Preparation and Cell Culture**—Male mice were maintained for 5 days on a low magnesium diet (ICN diet number 902205, Nutritional Biochemicals) supplemented with 0.05% MgSO\textsubscript{4} that is comparable with commercial mouse chow (17).

COS7, mouse distal convoluted tubule (MDCT), HEK293, and primary hippocampal neuronal cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate (110 mg/liter), 5 mm l-glutamine, 50 units/ml penicillin, and 50 \( \mu \)g/ml streptomycin in a humidified environment of 5% CO\textsubscript{2}-95% air at 37 °C. Primary neurons were isolated from rat brains and cultured using methods similar to that described previously (18). Where indicated, subconfluent cells were cultured in nominally Mg\textsuperscript{2+}-free, normal Mg\textsuperscript{2+}, 0.08 mm, or high Mg\textsuperscript{2+}, 5.0 mm, medium ( Stem Cell Technologies) for either 3 or 16 h prior to harvest or processing for immunocytochemistry and performed at 340 and 385 nm (chopper speed set at 100 Hz) and emission at 505 nm. Results are presented as the 340/385 ratio, which reflects the intracellular Mg\textsuperscript{2+} concentration.

**Quantitative Analysis of NIPA1 Transcripts by Real-time Reverse Transcription-PCR**—PCR products were quantified continuously with AB7000\textsuperscript{TM} (Applied Biosystems) using SYBR Green\textsuperscript{TM} fluorescence according to the manufacturer’s instructions. The primer set of mouse NIPA1 was: 5’-CTGGTTGGACTTCTTTGGGAT-3’ (forward) and 5’-TCCAATGGCC-TGTTCCCTAA-3’ (reverse). The relative amounts of NIPA1 RNA were normalized to the mouse \( \beta \)-actin transcripts.

**Genomic Sequence Analysis**—The NIPA1 cDNA sequence was determined by standard methods. The full-length NIPA1 amino acid sequence is in the GenBank\textsuperscript{TM} data base (accession numbers: NP_653200 (human) and NP_705806 (mouse)). Protein motifs were identified using BLASTP and the Swiss-Prot data base. Membrane topology was predicted by the SOSUI program based on Kyte-Doolittle hydrophobicity analysis (23).

**Western Blot Analysis**—A rabbit polyclonal antibody, anti-NIPA1, was raised against the third extracellular loop of the final cleaved human NIPA1 protein using a synthetic peptide, NH\textsubscript{2}-AQQDILHNPNSSQRLAC-COOH (amino acid residues 207–222). Affinity-purified rabbit anti-human NIPA1 antibody was diluted in PBS (Tris-buffered saline, 20 mm Tris, 200 mm

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\( ^5 \) A. Goytain, R. M. Hines, A. El-Husseini, and G. A. Quamme, unpublished observations.
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NaCl, pH 7.6) containing 0.5% BSA at a final concentration about 0.7 μl/ml. Western analysis was performed by incubating the blots with anti-NIPA1 antibody overnight at 4 °C followed by three washes with PBS, 0.1% Tween 20, 10 min each. The blots were then incubated with 1/10,000 horseradish peroxidase-conjugated donkey anti-rabbit secondary (Sigma) antibody for 1 h. After washing three times with PBS/Tween 20, 10 min each, the blots were visualized with ECL (Amersham Biosciences) according to the manufacturer’s instructions.

Immunoﬂuorescence Confocal Microscopy—Coverslips of cultured cells were fixed at room temperature for 10 min in 2% paraformaldehyde. Cells were washed three times with phosphate-buffered saline containing 0.3% Triton X-100 (PBST) before each antibody incubation. The following primary antibodies were used: NIPA1, GM130 (a cis-Golgi matrix protein), EEA1 (early endosome antigen 1), Rab5 and Rab8 (GTP-binding proteins) that were raised in mouse (BD Transduction Laboratories). Alexa 350-, Alexa 488-, and Alexa 568-conjugated secondary antibodies were obtained from Molecular Probes. All antibody reactions were performed in blocking solution composed of 2% normal goat serum in PBST for 1 h room temperature. Alexa 350- and 568-conjugated phalloidin (Molecular Probes) were used to stain for actin in the indicated experiments to aid in delimiting peripheral membrane ruffles. Following staining, coverslips were then mounted on slides with Fluoromount-G glycerol-based mounting medium (Southern Biotechnology). Oocytes were mounted in OCT cryostat medium and flash-frozen in isopentane cooled in liquid nitrogen. Ten-μm-thick sections were cut through frozen oocytes and mounted directly onto superfrost plus slides (Fisher). Sections were fixed in −20 °C methanol and processed for immunohistochemistry using the NIPA1 primary antibody and anti-rabbit Alexa 488 secondary antibody.

All images were taken using a 63× water lens affixed to a Zeiss LSM 510 Meta microscope and AxiosVision (epifluorescent) or LSM 510 Meta (confocal) software. Cells were selected from 10–12 fields of view and used for assessment of co-localization of antibody staining.

RESULTS

NIPA1 cDNA Is a Magnesium-responsive Gene—With the knowledge that differential gene expression is involved with selective control of epithelial cell magnesium conservation, our strategy was to use microarray analysis to identify cDNAs that were up-regulated with low magnesium (15). As our objective was to identify novel transport proteins, we prioritized these candidates according to structural properties reported for hypothetical transporters. One of the selected cDNA fragments identified by an increase in transcript was NIPA2 (Mus musculus) hypothetical protein MNCb-2146, renamed Nipa2. A BLAST search of the GenBank™ data base was performed and another member of this family, NIPA1, was identified. As NIPA1 was not on the mouse Affymetrix MG U74 Bv2 and MG U74 Cv2 arrays (Affymetrix) used at the time of our initial microarray analysis, we first showed that the NIPA1 transcript, like NIPA2 mRNA, is regulated by magnesium using real-time RT-PCR. NIPA1 mRNA increased 3.2-fold in immortalized distal convoluted tubule, MDCT, epithelial cells, n = 3 independent preparations, cultured in low magnesium compared with normal cells confirming that NIPA1 is differentially regulated by magnesium.

Amino Acid Sequence of NIPA1—The human and mouse genomes each contain four members of the NIPA family, designated NIPA1-NIPA4. NIPA1 is the least similar member of the family even though it is located in tandem with NIPA2 on chromosome 15q. The human and mouse NIPA1 proteins were found to be 98% identical in amino acid sequence. They differ by a short run of alanines on the N-terminal end. The human NIPA1 and mouse NIPA1 exhibited between 36 and 43% identity to the other three respective human and mouse NIPA forms. Genes encoding close homologues (83–91% amino acid sequence identity) are present in the chicken, orangutan, and chimpanzee genomes, indicating that divergence of the NIPA1 from NIPA2, NIPA3, and NIPA4 forms was an early event in vertebrate evolution (Fig. 1A).

Hydrophobicity profiles using the SOSUI program predicted a secondary structure with nine predicted transmembrane domains, TMDs (Fig. 1B). Also illustrated are the mouse T39R and G100R mutation sites that correspond to the respective human T45R and G106R mutation sites located in the predicted TMD1 and TMD3, respectively (Fig. 1B). NIPA1 Mediates Mg\(^{2+}\) Transport in Expressing Xenopus Oocytes—To determine whether NIPA1 encodes a functional Mg\(^{2+}\) transporter as does NIPA2, we prepared mouse NIPA1 cRNA, injected it into Xenopus oocytes and measured Mg\(^{2+}\)-evoked currents using two-microelectrode voltage clamp analysis and Mg\(^{2+}\) flux using mag-fura-2 fluorescence methodologies. The electrophysiological data gave evidence for a rheogenic process with inward currents in NIPA1 CRNA-injected oocytes, whereas there were no appreciable currents in control H\(_2\)O- or total poly(A)\(^+\) RNA-injected cells from the same batch of oocytes. Fig. 1C shows mean current-voltage (I-V) plots. There was a mean +28 mV shift in reversal potential with a decade increase in magnesium concentration, which approximated the theoretical value predicted by the Nernstian relationship. Among the inherent properties of all transporters is the property of substrate saturation. The Mg\(^{2+}\)-evoked currents, measured at a fixed time point, were saturable (Fig. 1D) demonstrating a Michaelis constant (K\(_m\)) of 0.69 ± 0.21 mM (Fig. 1D inset) that was independent of the larger negative clampings voltages (data not shown). Mag-fura-2 fluorescence determinations confirmed that the observed currents were due to Mg\(^{2+}\) influx (Fig. 1E). External magnesium increased the emission ratio of 340/385 excitation following voltage clamp at −70 mV. Ca\(^{2+}\) was not transported at rates necessary to change the fluorescence intensity of the dye and not at rates required to account for the concurrently measured currents.

Immunofluorescence using a specific anti-NIPA1 antibody shows predominantly surface localization of NIPA1 protein in NIPA1-expressing oocytes, whereas there was no staining in control, water-injected oocytes (Fig. 1F).

The second property of most transporters is substrate selectivity. Accordingly, a variety of extracellular divalent cations were used to determine the selectivity of the expressed NIPA1 channel. NIPA1 was relatively selective for Mg\(^{2+}\) (supplemen-
The reversal potential ratio of Sr\(^{2+}\)-evoked currents was notably less than Mg\(^{2+}\) and the ratio of other divalent cations tested, Ca\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\), were small compared with those evoked by Mg\(^{2+}\). In the experiments shown, currents were corrected for changes in membrane resistance caused by the respective divalent cation.

**FIGURE 1.** Molecular characterization of NIPA1. A, phylogenetic tree constructed from a multiple alignment of the following Homo sapiens (h), Mus musculus (m), Rattus norvegicus (r), Gallus gallus (g), Pan troglodytes (pt), Pongo pygmaeus (p), Bos taurus (b), and Canis familiaris (c) sequences using ClustalX version 1.88 (21) and the neighbor-joining method of Saitou and Nei (22) using Phylo Draw, version 0.8 (Graphics Application Laboratory, Pusan National University). B, predicted secondary structure of mouse NIPA1. The two mouse mutation sites, T39R and G100R, corresponding to the respective human T45R and G106R mutations are illustrated. C, current-voltage (I-V) relationships obtained from linear voltage steps from -150 mV to +25 mV in the presence of Mg\(^{2+}\)-free solutions or those containing the indicated concentrations of MgCl\(_2\). Oocytes were clamped at a holding potential of -15 mV and stepped from -150 mV to +25 mV in 25-mV increments for 2 s at each of the concentrations indicated. Shown are average I-V curves obtained from control H\(_2\)O-injected (n = 3) or NIPA1-expressing (n = 3-6) oocytes. Note the positive shift in reversal potential with increments in Mg\(^{2+}\) concentration. Values are mean ± S.E. of observations measured at the end of each voltage sweep for the respective Mg\(^{2+}\) concentration. D, summary of concentration-dependent Mg\(^{2+}\)-evoked currents in NIPA1-expressing oocytes using a holding potential of -125 mV. Mean ± S.E. values are those given in Fig. 1C. The Michaelis constant determined with nonlinear regression analysis was 0.69 mM. The Michaelis constant was independent of the respective holding potential. E, Mg\(^{2+}\) flux into NIPA1-expressing oocytes. Mag-fura-2 fluorescence ratios were measured in control and NIPA1-expressing oocytes, at resting potentials, in solutions consisting of nominally magnesium-free solutions and then with 2.0 mM MgCl\(_2\) with interruption as indicated. Oocytes were subsequently voltage-clamped at a holding potential of -70 mV, where indicated. Mg\(^{2+}\) fluxes were determined with fluorescence using the Mg\(^{2+}\)-sensitive dye, mag-fura-2. CaCl\(_2\), 2.0 mM, was added and removed where indicated. Results are presented as the 340/385 excitation ratio that reflect changes in divalent cation concentration. Results are mean of tracings performed with three different oocyte preparations. F, surface expression of NIPA1 protein in X. laevis oocytes determined with immunofluorescence. Left panel, control water-injected oocytes tested with affinity-purified NIPA1-specific antibody. The arrows indicate membrane surface (×200 magnification). Right panel, NIPA1-injected oocyte treated with NIPA1 antibody showing intense surface staining. The measured current for this oocyte was 0.12 μA with 2.0 mM external MgCl\(_2\) concentration clamped at -70 mV.
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using values from H\(_2\)O-injected oocytes (supplemental Fig. S1A). Accordingly, NIPA1-mediated cation transport was relatively selective for Mg\(^{2+}\).

Finally, a general property of transporters is the ability to be inhibited by related but not transported substrates. We tested if the cations that were not transported by NIPA1 would inhibit Mg\(^{2+}\)-evoked currents. Relatively large concentrations of 0.1 mM Mn\(^{2+}\) and 5.0 mM Ca\(^{2+}\) were tested in the presence of 2.0 mM MgCl\(_2\) (Fig. S1B). Mn\(^{2+}\) completely inhibited Mg\(^{2+}\) currents, whereas Ca\(^{2+}\) did not inhibit transport as reflected by the change in reversal potential for Mg\(^{2+}\). On balance, these data indicate that NIPA1-mediated transport demonstrates saturation, selectivity, and the ability to be differentially inhibited by other divalent cations.

**Tissue Distribution of Endogenous NIPA1 Protein Expression**—Using real-time RT-PCR, we show that NIPA1 transcripts are widely expressed among the mouse tissues tested. Western blot analysis was then carried out to determine the endogenous NIPA1 protein expression in the various mouse tissues (Fig. 2, left). For detection of NIPA1 protein, a specific antibody was generated by immunization of rabbits. There was an evident band at the expected molecular mass of 34 kDa in heart, kidney, liver, and colon, a faint band of 34 kDa in the brain, and no signal in the small intestine. A doublet was evident in the heart and kidney tissues that may suggest posttranslational modification in these cells. Additionally, there were faint bands in the heart and kidney tissues at about 68 kDa and a very strong band in brain that may represent the dimer of the protein. The latter was consistent for three separate tissue preparations.

As the NIPA1 mRNA is responsive to magnesium, we then determined NIPA1 protein expression in cells cultured in low magnesium media relative to normal magnesium. Using the mouse kidney cell line, MDCT, as a prototypic epithelial cell, Western blot analysis revealed about a 2-fold increase in protein density in cells grown in low magnesium (Fig. 2, right). Interestingly, the predominant increase was in the 68-kDa band with smaller increases in the 34-kDa signal. The larger band, perhaps a multimer of the NIPA1 protein, may be the active transporter.

**Subcellular Localization of Endogenous NIPA1 Protein**—To investigate the subcellular localization of NIPA1 protein, we performed immunofluorescence using the specific anti-NIPA1 antibody in kidney COS7 cells. NIPA1 extensively co-localized with EEA1 and Rab5 that are early endosome proteins (Fig. 3, A and B). Furthermore, the punctate peripheral staining pattern of NIPA1 is consistent with the presence of NIPA1 in early endosomes. To conclusively demonstrate the localization of NIPA1 to early endosomes, a constitutively active form of Rab5, Rab5Q79L, was used. NIPA1 co-localized with the expressed Rab5Q79L in enlarged endosomes (Fig. 3B). In addition to the endosomal localization, NIPA1 staining was widely dispersed across the cell surface at the membrane ruffles as evidenced by phaloidin staining of actin (Fig. 3C). Omission of the primary antibody resulted in the complete absence of immunostaining.

**FIGURE 2. Tissue distribution of endogenous mouse NIPA1 protein expression.** Left, Western blotting of endogenous NIPA1 protein in various mouse tissues. An aliquot of protein (50 μg) from the indicated mouse tissues was applied to each lane and labeled with a specific polyclonal antibody for NIPA1. The gel is representative of three separate tissue preparations. Right, NIPA1 protein increases in epithelial cells cultured in low magnesium media. Shown is a representative blot, one of seven performed on different cell preparations. The mean band density increased 163 ± 7% with low magnesium relative to those cultured with normal concentrations of magnesium.
(data not shown). As endogenous NIPA1 was detected with the antibody, this pattern of distribution was not the inefficient targeting of overexpressed protein resulting from heterologous expression. This pattern of distribution is consistent with the notion that NIPA1 translocates through the early (and likely also recycling) endosomes and the plasma membrane. Results from the COS7 cells are presented here because the intracellular organelle compartmentalization was easier to discern with confocal microscopy; however, similar patterns of distribution were observed with kidney HEK293 and MDCT cells (data not shown) and primary hippocampal neuronal cells (supplemental Fig. S2). NIPA1 was evident in a punctate pattern in the soma and dendrites of the cultured neurons and was absent from synaptic sites (supplemental Fig. S2). The suggestion that endogenous NIPA1 is present at the surface membrane is in keeping with the functional studies performed with heterologous-expressing oocytes.

Changes in Extracellular Magnesium Lead to Subcellular Redistribution of NIPA1 Protein—If NIPA1 is involved with cellular magnesium homeostasis, we would expect that there may be subcellular redistribution of protein in response to changes in external magnesium concentration. COS7 cells were cultured in relatively high magnesium, 5.0 mM MgCl₂, for 3 or 12 h or in nominally magnesium-free medium for 3 or 12 h, and subcellular localization was determined with immunofluorescence as given above. The most evident changes in NIPA1 subcellular distribution occurred within the surface membrane and the early endosomes (Fig. 4A). NIPA1 was modestly reduced in the peripheral membrane following culture in high Mg²⁺. Relative to those cells grown in normal culture media containing 0.5 mM magnesium, NIPA1 protein was reduced at the surface membrane and increased in the early endosomes (Fig. 4B). This apparent trafficking from the surface to the early endosome compartment was noticeable at 3 h and marked at 12 h following the change to high magnesium medium. The accumulation of NIPA1 protein was large enough to appear as “aggregation” of protein in the early endosomes (supplemental Fig. S3A).

Placing the cells in low magnesium for 3 and 12 h led to an increase in NIPA1 protein in the early endosome pool but not aggregation rather it was more punctate within the enlarged endosomes compared with that observed with normal magnesium (supplemental Fig. S3A). Moreover, there was a marked recruitment of NIPA1 protein to the surface membrane that was apparent at 3 h and marked at 12 h after removal of magnesium. Indeed, the surface labeling with NIPA1 was so extensive that the cell outline was evident suggesting that it was localizing in the plasma membrane (indicated in Fig. 4B).

Loss-of-function Mutations of NIPA1 Protein—Rainer et al. (9) identified a nucleotide substitution at position 134 of the NIPA1 cDNA that resulted in an amino acid substitution at position 45 of the NIPA1 protein (T45R) in a HSP kindred. This site corresponds to position Thr39 of the mouse NIPA1. The T39R mutation occurs at a conserved residue at the interface of the first transmembrane domain (TMD1) and the first putative loop (Fig. 1B). This would be expected to extend the first TMD by three amino acids that may alter protein trafficking or transport function. To test this, we replaced Thr39 (ACG) with Arg39 (AGG) and injected the associated cRNA into oocytes and performed voltage clamp and fluorescence analysis. The T39R mutation led to diminished Mg²⁺ transport by about 30% compared with the wild-type NIPA1 as determined by both electrophysiology (Fig. 5A) and mag-fura-2 fluorescence (Fig. 5B). This result could be caused by either a failure of transporters to correctly traffic to the plasma membrane or by transporters that traffic normally but are non-functional. To distinguish between these possibilities, the wild-type NIPA1 and the mutant NIPA1T39R were fused with HA so that cell surface expression could be evaluated in transiently transfected COS7 cells by immunofluorescence techniques. The wild-type NIPA1-HA was expressed in the early endosomes as well as the cell surface in a similar fashion as was seen with immunofluorescence with the NIPA1 antibody (Fig. 5C). The plasma membrane NIPA1T39R-HA was noticeably less than wild-type NIPA1-HA-transfected cells, and there was significant retention of the mutant construct in the perinuclear network-like structure comprising the endoplasmic reticulum (Fig. 5D). Erroneous and misfolded proteins are identified by endoplasmic reticulum quality control. There was also an apparent
increase in endosomal size that might suggest abnormal protein processing within the endosomes (Fig. 5E).

Reed (10), Chen (11), Munhoz (12), and their respective colleagues have identified a missense substitution, G106R, in four large unrelated families with HSP. The human G106R mutation corresponds to mouse G100R (Fig. 1B). This mutation is located in a highly conserved amino acid region within the central portion of the third TMD so that it would be expected to significantly alter the structure of the protein (10). We replaced Gly100 (GGG) with Arg100 (AGG), and the associated cRNA was injected into oocytes. The G100R mutation completely abrogated Mg\(^{2+}\)/H11001 transport as determined by electrophysiology or mag-fura-2 fluorescence (Fig. 5, A and B). In concert with these findings, the NIPA1G100R-HA was retained in the endoplasmic reticulum (ER) relative to the wild-type NIPA1 protein. E, increase in endosomal size of NIPA1T39R-HA and NIPA1T100R-HA transfected relative to the wild-type NIPA1-HA-transfected COS7 cells. WT, wild type.
NIPA1T38R and the absence of NIPA1G100R protein results in the partial and complete loss of Mg\(^{2+}\) uptake, respectively, of these two mutant forms.

**DISCUSSION**

We show here that NIPA1, the basis of some forms of HSP, mediates Mg\(^{2+}\) transport. The evidence that NIPA1 is a magnesium transporter is persuasive. First, expression of NIPA1 in *Xenopus* oocytes produces Mg\(^{2+}\)-evoked currents with channel-like properties measured with voltage-clamp conditions. The reversal potential shifts to the right with a magnitude of 28 mV as predicted by the Nernst relationship with decade increases in magnesium concentration. Mg\(^{2+}\) currents are concentration-dependent, saturable, reversible, and inhibitable as would be expected of a channel-like transporter. Second, NIPA1 mediates Mg\(^{2+}\) flux as determined by fluorescence with the Mg\(^{2+}\)-sensitive mag-fura-2 dye. Third, NIPA1 transcript and protein are quantitatively altered with changes in Mg\(^{2+}\) concentration, consonant with our initial paradigm (16). Fourth, we demonstrate that NIPA1 trafficking to the early endosomes and plasma membrane is increased with diminished extracellular Mg\(^{2+}\), as would be expected of a magnesium-regulated transporter. Finally, we show that the NIPA1T38R and NIPA1G100R mutations lead to a loss of Mg\(^{2+}\) transport in oocytes expressing the altered NIPA1. We conclude that NIPA1 encodes a Mg\(^{2+}\) transporter. What is not clear is how a NIPA1 loss-of-function can give rise to a network hyperexcitability that is able to cause spastic paraplegia.

Rainer et al. (9) and Chai et al. (5) have reported the presence of two NIPA1 transcripts, 1.9–2.2 and 7.5 kb, in human and mouse (5, 9). The alternative mRNA isoforms arise from alternative polyadenylation sites within exon 5 (5). The two RNAs are constitutively expressed in all tissues tested but are especially enriched in brain tissue (5, 9). Our results using Western analysis are consistent with these earlier observations. Using a specific polyclonal antibody that we generated in rabbits, we show here that the endogenous NIPA1 protein is present in many tissues but particularly abundant in brain. Interestingly, the major form in brain cells appears to be about twice the molecular size predicted from the amino acid sequence that suggests that it may be a dimer. Many membrane receptors, transporters, and integral membrane proteins require dimerization or higher oligomerization for their activity.

In response to low magnesium, there was a 65% increase in NIPA1 protein in mouse kidney cells. Moreover, the increase was principally in the 68-kDa form with much smaller increments in the 34-kDa size. We speculate that the active form of the NIPA1 transporter is the larger dimerized protein species. It is abundantly expressed in epithelial cells cultured in low magnesium that is associated with elevated Mg\(^{2+}\) transport. Based on the observation that the form in the normal brain cell is primarily the larger 68-kDa size, we further speculate that the NIPA1 transporter is constitutively active in neural tissue.

Within the cell, the NIPA1 protein was principally localized in the early endosomal compartment and the peripheral surface. There was very little protein found in the nucleus, endoplasmic reticulum, Golgi, or the late endosomes and lysosomes. This localization suggests that the NIPA1 protein plays a role at the surface membrane. In support of this notion is the redistribution of NIPA1 in response to magnesium. There was an apparent trafficking from the surface and aggregation in the endosomes in cells cultured in high magnesium, whereas there was a marked movement of NIPA1 to the periphery with low magnesium. Consonant with the Western analysis, there was a noticeable increase in total protein in epithelial cells grown in low magnesium and an apparent clustering of NIPA1 protein in early endosomes giving a punctate appearance. We conclude that high magnesium leads to diminished surface expression of NIPA1 protein and sequestration in endosomes whereas low magnesium results in an increase in early endosomal NIPA1 protein and enhanced targeting to the surface membrane. These changes in protein expression likely account, in part, for the associated changes in Mg\(^{2+}\) transport.

Finally, we show that the NIPA1T38R and NIPA1G100R mutations lead to a loss of Mg\(^{2+}\) transport in oocytes expressing the altered NIPA1. The missense mutations G100R and T45R lead to a loss-of-function suggesting that G100 and T45 sites are important, if not essential, in processing of the NIPA1 Mg\(^{2+}\) transport protein. Moreover, Gly\(^{106}\) and Thr\(^{45}\) are conserved among ortholog NIPA1 channels supporting the notion that the mutation is strongly pathogenetic. More recently, there has been another mutation, A100T, reported in a family presenting with the HSP phenotype (19). The changes in transport remain to be determined. There are no similar consensus sites in the other members of the NIPA family, either the paralog NIPA2 or the related NIPA3 and NIPA4, so that the protein folding is likely different between these transporters. The significance of these differences in the biophysical functioning of the NIPA transporters remain to be examined. It is also interesting that G106R leads to complete loss-of-function whereas T45R mediates some Mg\(^{2+}\) transport. It would be interesting to see if there are differences in clinical symptoms between these two groups of HSP patients.

The present findings show that NIPA1 forms a Mg\(^{2+}\) transporter. How a defective Mg\(^{2+}\) transporter would lead to the phenotype of HSP is unclear. In addition to the progressive lower limb spasticity, some subjects with uncomplicated HSP have urinary urgency and mild vibratory sensation impairment in the toes (9). To our knowledge, disturbances in magnesium metabolism in these individuals have not been reported nor has the effect of magnesium supplementation been assessed. Also of interest is why NIPA1-linked HSP patients present with symptoms in their adolescent or adult years rather than infancy or childhood (6). It may suggest an age-related or temporal control of NIPA1 gene expression. Moreover, because HSP is a clinically and genetically heterogenous group of neurodegenerative disorders, it is likely that numerous other factors and genes can contribute to the generation of the seizure phenotypes observed in the HSP spectrum (20). Using magnetic resonance imaging, Hedera et al. (20) have recently shown that early and severe spinal cord atrophy occurs in NIPA1-HSP patients due to axonal loss suggesting degeneration of the descending spinal cord tracts innervating the lower extremities. Interestingly, analysis of the brain did not reveal any constant abnormalities that would distinguish the NIPA1 mutation HSP patients from the control group. Accordingly, it is difficult to
speculate on the function of NIPA1 in the brain and spinal cord or other nonneural cells where it is found.

We conclude from the present studies that NIPA1 normally encodes a Mg\(^{2+}\)/H\(^{+}\) transporter and the loss-of-function of NIPA1(SPG6) due to mis trafficking of the mutated protein provides the basis for the HSP phenotype.

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