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Identification and Characterization of Wolframin, the Product of the Wolfram Syndrome Gene (*WFS1*), as a Novel Calmodulin-Binding Protein†

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ABSTRACT: To search for calmodulin (CaM) targets, we performed affinity chromatography purification of a rat brain extract using CaM fused with GST as the affinity ligand. Proteomic analysis was then carried out to identify CaM-binding proteins. In addition to identifying 36 known CaM-binding proteins, including CaM kinases, calcineurin, nNOS, the IP_3 receptor, and Ca^{2+}-ATPase, we identified an ER transmembrane protein, wolframin [the product of the Wolfram syndrome gene (*WFS1*)] as interacting. A CaM overlay and an immunoprecipitation assay revealed that wolframin is capable of binding the Ca^{2+}/CaM complex *in vitro* and in transfected cells. Surface plasmon resonance analysis and zero-length cross-linking showed that the N-terminal cytoplasmic domain (residues 2−285) of wolframin binds to an equimolar unit of CaM in a Ca^{2+}-dependent manner with a K_D for CaM of 0.15 μM. Various truncation and deletion mutants showed that the Ca^{2+}/CaM binding region in wolframin is located from Glu90 to Trp186. Furthermore, we demonstrated that three mutations (Ala127Thr, Ala134Thr, and Arg178Pro) associated with Wolfram syndrome completely abolished CaM binding of wolframin. This observation may indicate that CaM binding is important for wolframin function and that impairment of this interaction by mutation contributes to the pathology seen in Wolfram syndrome.

Changes in the concentration of intracellular calcium ion can lead to various cellular events such as secretion, contraction, cell division, and gene expression (*1*). Calmodulin (CaM)[1] has been shown to play an important role in physiological responses in many tissues and cells and to mediate a number of actions of Ca^{2+} as an intracellular second messenger (*2*, *3*). CaM interacts with many cellular targets to regulate their function in response to an increasing concentration of intracellular Ca^{2+}, resulting in modulation of a large number of Ca^{2+}-dependent signal transduction processes (*4*). There are many cellular CaM targets as diverse as enzymes, including protein kinases (*5*, *6*), phosphatase (*7*), adenylate cyclase (*8*), and phosphodiesterase (*9*), cytoskeletal proteins, including motor proteins [unconventional myosin (*10*)], muscle proteins [caldesmon (*11*), calponin (*12*), and dystrophin (*13*)], actin-binding protein [synapsin (*14*), adducin (*15*), and myristoylated alanine-rich C kinase substrate (MARCKS) (*16*)], and membrane proteins [Ca^{2+}-ATPase (*17*) and IP_3 receptor (*18*)].

Recently, tandem mass spectrometry (MS/MS) was used to identify 140 putative Ca^{2+}/CaM-binding proteins in a mouse brain cytosolic fraction that had been purified using CaM affinity chromatography (*19*). The single-step enrichment of CaM-binding proteins from crude tissue extracts by a CaM-coupled affinity matrix (*20*) is a key step for the comprehensive identification of CaM targets. CaM is ubiquitously expressed and highly abundant in most tissues, especially in brain (~0.5 mg/g of tissue) (*21*). When brain extracts are applied onto CaM-Sepharose in the presence of Ca^{2+}, many CaM-binding proteins likely interact with highly concentrated endogenous CaM before they are trapped with the CaM-coupled affinity matrix. While incubating the tissue extracts for some time with CaM-Sepharose may give more time for dissociation of

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[1]Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; GST, glutathione *S*-transferase; nNOS, neuronal nitric oxide synthase; MARCKS, myristoylated alanine-rich C kinase substrate; IP_3 receptor, inositol triphosphate receptor; LC, liquid chromatography; MS/MS, tandem mass spectrometry; SPR, surface plasmon resonance; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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target proteins from the endogenous CaM to take place, the efficiency of recovery of target proteins bound to CaM-Sepharose depends on the ratio of the amount of endogenous CaM versus that of immobilized CaM. Therefore, it might be difficult to identify some CaM targets present in rare amounts in the cells using single-step CaM-Sepharose chromatography, although abundant CaM-binding proteins such as cytoskeletal proteins and protein kinases should be easily detected.

Here, to search for novel CaM targets, we attempted to improve single-step affinity purification of CaM targets using recombinant CaM fused with GST as the affinity ligand. Then, CaM targets were eluted from the affinity ligand and identified using mass spectrometry. By using this functional proteomic approach, we identified wolframin from rat brain, which is the known product of the Wolfram syndrome gene (*WFS1*) (*22*), as a novel CaM-binding protein. Mutations in the coding region of the *WFS1* gene cause Wolfram syndrome, which is the association of juvenile onset diabetes mellitus and optic atrophy, also known as the DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) syndrome (*23*−*25*). Here, we characterized CaM binding of wolframin and demonstrated that three distinct mutations in the cytoplasmic domain of wolframin, which are associated with Wolfram syndrome, impaired its CaM binding ability.

EXPERIMENTAL PROCEDURES

Materials. Recombinant rat CaM was expressed in *Escherichia coli* BL21(DE3) (Stratagene) using the pET-CaM vector (kindly provided by N. Hayashi, Fujita Health University, Toyoake, Japan) and was purified by phenyl-Sepharose column chromatography (*26*). A recombinant rat CaMKI α isoform was expressed in *E. coli* JM-109 as previously described (*27*). Rat CaM fused with GST by a Gly×6 spacer (CaM−GST) was constructed as follows. A cDNA-encoded rat CaM with Gly×3 at its C-terminus was amplified by PCR with PrimeSTAR HS DNA polymerase (Takara, Tokyo, Japan) using pET-CaM as the template with a sense primer (5′-GGCCCACCATGGCTGACCAACTGACTGA-3′) and an antisense primer (5′-ACCACCACCCTTCGCTGTCATCATTTGTAC-3′), and a cDNA-encoded GST with Gly×3 at its N-terminus was amplified using pGEX-2T (GE Healthcare UK, Ltd., Buckinghamshire, U.K.) as a template with a sense primer (5′-GGTGGTGGTATGTCCCCTATACTAGGTTAT-3′) and an antisense primer (5′-GGCTCGAGTCAGGATCCACGCGGAACCAG-3′). The cDNAs were digested with *Nco*I and *Xho*I, respectively, and ligated into the *Nco*I/*Xho*I site in vector pET16b (Novagen). The recombinant CaM−GST protein was expressed in *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene), followed by purification using glutathione-Sepharose chromatography and subsequent purification with phenyl-Sepharose chromatography. For construction of the FLAG-tagged CaM (FLAG-CaM) expression vector, a PCR fragment encoding Ala1−Lys148 of rat CaM was ligated into the pME-FLAG vector. Anti-HA antibody (12CA5) and anti-FLAG antibody (clone M2) were obtained from Roche Applied Science (Indianapolis, IN) and Sigma-Aldrich (St. Louis, MO), respectively. Biotinylated CaM was purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from standard commercial sources.

Affinity Chromatography. Whole rat brains (5.3 g) were homogenized with 20 mL of buffer A [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin] containing 1 mM EDTA, 1 mM EGTA, and 1% NP-40, followed by centrifugation at 100000*g* for 60 min at 4 °C. Purified CaM−GST protein (19 mg) was added and mixed with the supernatant, followed by addition of $CaCl_2$ (final concentration of 5 mM). The mixture of rat brain extract with CaM−GST protein was applied to glutathione-Sepharose columns (2 mL bed volume, GE Healthcare UK, Ltd.), and then the columns were washed with 20 mL of buffer A containing 0.2 mM $CaCl_2$ (buffer B). After being washed with 20 mL of buffer B containing 1 M NaCl, the column was washed with 20 mL of buffer B. Elution of CaM-binding proteins from the column was carried out using buffer A containing 2 mM EGTA. Fractions (2 mL) were collected; SDS−PAGE sample buffer (36 μL) and 1 M DTT (4 μL) were added to the eluate, and then each sample was stored at −80 °C until it was analyzed by mass spectrometry or CaM overlay.

Mass Spectrometry Analysis. A 24 μL sample of the eluate (fraction 2, shown in Figure 1B) from the CaM−GST coupled glutathione-Sepharose column described above was concentrated and then separated by SDS−10% PAGE and lightly stained with Coomassie Brilliant Blue. Next, 20 gel slices were excised from the sample lane in the range from ∼30 to >200 kDa, followed by in-gel digestion with 10 μg/mL trypsin (Promega, Madison, WI) overnight at 37 °C (*28*). The digested peptides were eluted with 0.1% formic acid and were subjected to LC−MS/MS analysis, which was performed on a Q-Tof 2 quadrupole/time-of-flight hybrid mass spectrometer (Micromass, Manchester, U.K.) interfaced with a CapLC capillary reverse-phase liquid chromatography system (Micromass). A 90 min linear gradient from 5 to 45% acetonitrile in 0.1% formic acid was produced and split at a 1:20 ratio; the gradient solution was then injected into a NanoLC column (PepMap C18, 75 μm × 150 mm; LC Packings, Sunnyvale, CA) at a rate of 100 nL/min. The eluted peptides were sprayed directly into the mass spectrometer. MS/MS data were acquired with MassLynx (Micromass) and converted to a single text file (containing the observed precursor peptide *m*/*z*, the fragment ion *m*/*z*, and intensity values) with ProteinLynx (Micromass). The file was analyzed using the Matrix Science Mascot MS/MS Ion Search (http://www.matrixscience.com) to search and assign the obtained peptides to the NCBI nonredundant database. We set the search parameters as follows: database, NCBInr; taxonomy, all; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tolerance, ±0.2 Da; and MS/MS tolerance, ±0.2 Da.

Cloning and Construction of Rat Wolframin cDNAs. Rat wolframin cDNA (GenBank accession number AF136378) was obtained by reverse transcriptase-mediated PCR (RT-PCR) with PrimeSTAR HS DNA

polymerase (Takara) using rat brain cDNA (QUICK-Clone, Clontech Laboratories, Inc., Mountain View, CA) as a template with a sense primer (5′-CAA-AATGGCGCCTTACAGCCTACTGGTCAC-3′) and an antisense primer (5′-ACCGAGGCGTCCTCCA-GTGGCAGACACTCC-3′) derived from the genomic sequence on rat chromosome 14. The initial PCR was followed by a second PCR using sense primer 5′-AGCTCAGGCACCCCACCTCCGAGCCCCTCT-3′ and antisense primer 5′-GGTCTAGATCAGGCGGCA-GACAGGAATGGG-3′. The PCR fragments were subcloned into the *Eco*RV/*Xba*I site of the pME18s-HA vector. For expression of GST−wolframin 2−285, a PCR fragment encoding Ser2−Pro285 of wolframin (sense primer 5′-CCTCTAGACAGCTCAGGCA-CCCCACCTCCG-3′ and antisense primer 5′-TGG-CAGGTCCTCGGGGCTCTTCCCTGACAG-3′) was ligated into the *Xba*I/*Sma*I site of vector pGEX-KG-PreS-His_6, resulting in the addition of GHHHHHH at the C-terminal end (after Pro285) of GST−wolframin 2−285 (pGEX-KG-PreS-wolframin 2−285-His_6). A deletion mutant of HA-wolframin lacking residues 2−161 (Δ2−161), C-terminal truncation and N-terminal deletion mutants of GST−wolframin 2−285, Ala127Thr, Ala134Thr, and Arg178Pro mutants of HA-wolframin, and GST−wolframin 2−285 were created by PCR with PrimeSTAR HS DNA polymerase or site-directed mutagenesis (GeneEditor system, Promega) using wild-type pME18s-HA-wolframin as the template. The nucleotide sequences of all constructs used in this study were confirmed by sequencing on an ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA).

Purification of Recombinant GST−Wolframin 2−285. GST−wolframin 2−285 wild-type and mutant cDNAs (pGEX-KG-PreS-wolframin 2−285-His_6) were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene), and expression of the recombinant proteins was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. An *E. coli* pellet containing GST fusion protein was lysed with PBS followed by purification using glutathione-Sepharose column chromatography as described in the manufacturer's protocol. Further purification was then carried out using Ni-NTA agarose column chromatography (Qiagen) to obtain GST-fused proteins containing wolframin 2−285. The cytoplasmic domain of wolframin (residues 2−285) was prepared by digestion of purified recombinant GST−wolframin 2−285 with PreScission Protease (GE Healthcare UK, Ltd.), followed by removal of digested GST or undigested GST−wolframin 2−285 with glutathione-Sepharose.

Expression of HA-Wolframin in COS-7 Cells. COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfection of pME-HA-rat wolframin wild-type or mutant constructs (10 μg) into COS-7 cells (10 cm dishes) was carried out using Lipofectamine reagent (Invitrogen, Carlsbad, CA). After incubation for 40 h, the cells were washed with PBS and then collected with buffer C [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL trypsin inhibitor] followed by sonication. Cell extracts were centrifuged at 22140g for

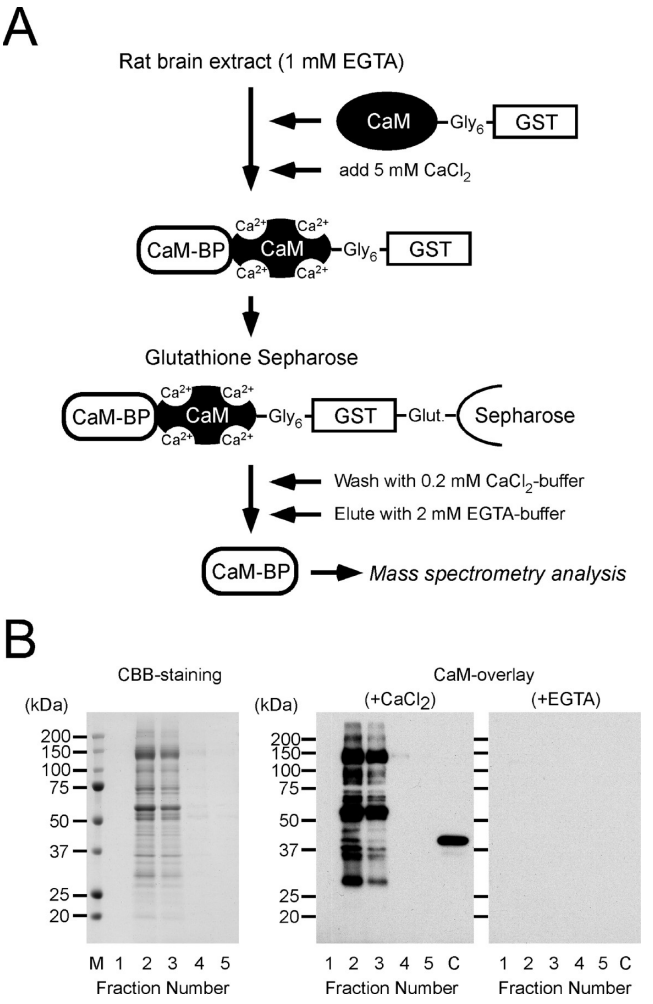

FIGURE 1: Functional proteomic approach to identifying potential targets for CaM. (A) Protocol of the functional proteomic approach to identifying CaM-binding proteins. Recombinant CaM−GST protein was added to rat brain extract prepared with 1 mM EGTA-containing buffer. After addition of 5 mM $CaCl_2$, glutathione-Sepharose was added to the sample followed by extensive washing with 0.2 mM $CaCl_2$ buffer. CaM-binding proteins were eluted by addition of 2 mM EGTA containing buffer. After the eluted sample was separated by SDS−10% PAGE, 20 gel slices were excised from the sample lane in the range from ~30 to > 200 kDa, followed by in-gel digestion with trypsin. The eluted peptides were then analyzed by LC−MS/MS as described in Experimental Procedures. (B) EGTA eluate (fractions 1−5) analyzed by SDS−PAGE followed by either Coomassie Brilliant Blue staining (left) or CaM overlay in the presence of 1 mM $CaCl_2$ (center) or 2 mM EGTA (right) as described in Experimental Procedures. Lane M in the left panel shows the molecular mass marker. Lane C in the center and right panels shows recombinant CaM-KI as a positive control.

15 min at 4 °C, and then the precipitated membrane fraction was dissolved with 50 μL of SDS−PAGE buffer, followed by CaM overlay analysis or Western blot analysis using a 5 μL sample as described above.

Immunoprecipitation. Transfection of pME-HA-rat wolframin wild-type (5 μg) with or without pME-FLAG-CaM (5 μg) into COS-7 cells (10 cm dishes) was conducted as described above. After being incubated for 40 h, the cells were washed with 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl and then collected with buffer D [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL trypsin inhibitor] in the presence of either

Table 1: Identified CaM-Binding Proteins from Rat Brain[a]

NCBI entry	molecular weight	protein name	no. of peptides (% coverage)
gi|19745200	41612	calcium/calmodulin-dependent protein kinase I α	4 (12)
gi|8393035	38415	calcium/calmodulin-dependent protein kinase I β	4 (14)
gi|6978593	54081	calcium/calmodulin-dependent protein kinase II α	10 (24)
gi|108796657	60423	calcium/calmodulin-dependent protein kinase II β	6 (16)
gi|19424316	59001	calcium/calmodulin-dependent protein kinase II γ	4 (10)
gi|77404384	53118	calcium/calmodulin-dependent protein kinase IV	13 (34)
gi|13928916	55873	calcium/calmodulin-dependent protein kinase kinase α	9 (18)
gi|13786172	64405	calcium/calmodulin-dependent protein kinase kinase β	8 (17)
gi|13027458	54072	CaM kinase-like vesicle-associated	12 (37)
gi|18034789	46648	phosphorylase kinase, γ 2	1 (2)
gi|8394030	58606	calcineurin A	16 (39)
gi|16258811	164254	neuronal nitric oxide synthase	38 (29)
gi|13591973	49947	inositol 1,4,5-trisphosphate 3-kinase A	4 (13)
gi|57977323	47239	2′,3′-cyclic nucleotide 3′-phosphodiesterase	16 (34)
gi|149022377	61972	phosphodiesterase 1A, calcium/calmodulin-dependent	12 (27)
gi|149031867	56596	phosphodiesterase 1B, calcium/calmodulin-dependent	6 (12)
gi|56188	35813	glyceraldehyde-3-phosphate dehydrogenase	11 (43)
gi|62825891	85289	phosphofructokinase	9 (13)
gi|1730559	96113	glycogen phosphorylase	4 (4)
gi|16758008	129428	plasma membrane calcium ATPase	18 (17)
gi|55925610	312397	inositol 1,4,5-triphosphate receptor	6 (2)
gi|223556	50210	tubulin α	14 (42)
gi|8850229	100423	microtubule-associated protein 6, STOP	34 (47)
gi|57620	198863	microtubule-associated protein 2	3 (2)
gi|31543764	284420	α-spectrin	29 (12)
gi|6978449	62748	adducin β	1 (2)
gi|266495	29777	myristoylated alanine-rich C-kinase substrate (MARCKS)	5 (30)
gi|9507159	73943	synapsin I	16 (36)
gi|112350	52422	synapsin IIb	11 (27)
gi|19424156	75893	A-kinase anchor protein 5	2 (4)
gi|19424162	75785	rabphilin 3A	15 (26)
gi|13928824	29103	14-3-3 ε	3 (12)
gi|4454315	18458	myelin basic protein	3 (19)
gi|51859516	83289	heat shock 90 kDa protein	11 (17)
gi|6681273	50422	elongation factor 1 α	5 (10)
gi|52138613	47757	Sjögren syndrome antigen B	1 (2)

[a] Rat brain proteins binding to Ca^{2+}/CaM−GST were purified and separated via SDS−PAGE, and then slices were cut from the gel, digested with trypsin, and identified using LC−MS/MS. The right column shows the number of different identified peptides with sequence coverage (%) within parentheses. Identified peptides and their positions are listed in Supplemental Data 1 of the Supporting Information.

2 mM EGTA or 2 mM $CaCl_2$, followed by sonication. Cell extracts were centrifuged at 15000 rpm for 15 min at 4 °C, and then the supernatant was precleared with 25 μL of Protein G-Sepharose (GE Healthcare UK, Ltd.). Precleared cell lysate was incubated with 10 μg of anti-FLAG antibody for 2 h and then incubated with 25 μL of Protein G-Sepharose overnight. After the immunoprecipitates had been washed with buffer D in the presence of either 2 mM EGTA or 2 mM $CaCl_2$, 50 μL of SDS−PAGE sample buffer was added to the sample followed by boiling for 10 min. Five microliters of the sample was analyzed by Western blotting with anti-HA and anti-FLAG antibodies.

CaM Overlay Assay. Samples were first separated via SDS−PAGE followed by transfer of the proteins to a nitrocellulose membrane (Hybond C, GE Healthcare UK, Ltd.). The membrane was then incubated with 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 5% bovine serum albumin in the presence of either 1 mM $CaCl_2$ or 2 mM EGTA for 1 h and then incubated with 0.5 μg/mL biotinylated CaM in the same buffer without bovine serum albumin for 1 h. After being extensively washed, the membrane was incubated with avidin-bound horseradish peroxidase in either $CaCl_2$- or EGTA-containing buffer for 1 h. After the membrane had been washed again, a chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA) was used for detection of the CaM binding signal.

Surface Plasmon Resonance Analysis. Binding interactions were assessed by surface plasmon resonance (SPR) using a Biacore 2000 system (Biacore AB, Uppsala, Sweden). The recombinant cytoplasmic domain of wolframin (residues 2−285) was covalently immobilized on CM5 research grade chips (Biacore) to a level represented by 1625 response units using *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-(dimethylamino)propyl]cabodiimide (EDC) (Biacore amine coupling kit). CaM (18−290 nM) was injected over the sensor surface at a flow rate of 30 μL/min in binding buffer (HBS-P) [10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% Surfactant P20] containing either 1 mM $CaCl_2$ or 2 mM EGTA. CaM was allowed to interact with the surface of the sensor chip for 2 min, after which CaM-free binding buffer was injected over the sensor surface to monitor CaM dissociation. The

sensor surface was regenerated using a calcium-free flow buffer (HBS-P containing 5 mM EGTA) between injections. BIAevaluation Software (version 4.1; Biacore) was used to process and analyze raw binding data to produce a K_D value for CaM.

Zero-Length Cross-Linking. Two-step zero-length cross-linking was carried out according to the procedure of Grabarek and Gergely (*29*). After CaM (40 μg) was treated with 50 mM NHS and 30 mM EDC at 25 °C for 15 min in a solution (40 μL) containing 150 mM NaCl, 25 mM HEPES (pH 7.5), and 0.2 mM $CaCl_2$, activation was terminated by addition of 2-mercaptoethanol (10 μL). Then, activated CaM (16 μg) was incubated with the recombinant cytoplasmic domain of wolframin (residues 2−285, 16 μg) in the presence of either 1 mM $CaCl_2$ or 4 mM EGTA at 25 °C for 1 h. After termination of the reaction via the addition of SDS−PAGE sample buffer, samples were analyzed by SDS−12.5% PAGE.

Other Methods. Western blot analysis was performed with horseradish peroxidase-conjugated anti-mouse (GE Healthcare UK, Ltd.) secondary antibody, and a chemiluminescence reagent (PerkinElmer Life Sciences) was used for detection. The protein concentration was estimated by staining the samples with Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin as a standard.

RESULTS

Identification of CaM Targets Using a Functional Proteomic Approach. It has been shown that CaM targets many cellular proteins to provide a wide range of Ca^{2+} signal transduction. To discover novel CaM-mediated signaling pathways, we performed comprehensive analysis of CaM-binding proteins using functional proteomics. First, we performed affinity purification of CaM targets using GST-fused CaM as an affinity ligand, followed by mass spectrometry analysis for identification of CaM-interacting proteins (Figure 1A). For the affinity ligand, we constructed and purified a rat CaM-fused GST (CaM−GST) with a spacer composed of six glycine residues between CaM and GST. Purified CaM−GST (19 mg) was added and mixed with rat brain extract that was prepared in the presence of 1 mM EGTA, followed by addition of 5 mM $CaCl_2$ to form a complex of Ca^{2+}-loaded CaM−GST with CaM-binding proteins from brain. After addition of glutathione-Sepharose to trap Ca^{2+}-loaded CaM−GST bound to CaM-binding proteins, extensive washing of the glutathione-Sepharose resin with a buffer containing a low concentration (0.2 mM) of $CaCl_2$ was carried out. Endogenous CaM-binding proteins were eluted with 2 mM EGTA-containing buffer from glutathione-Sepharose resin coupled with CaM−GST. The EGTA eluate from the CaM−GST-coupled resin was subjected to SDS−PAGE, followed by either protein staining (Figure 1B, left panel) or CaM overlay analysis in the presence or absence of Ca^{2+} (Figure 1B, center and right panels). The proteins with a molecular mass range of ~30−200 kDa that eluted from the resin appeared to specifically interact with Ca^{2+}/CaM, since we observed a CaM binding profile (Figure 1B, center panel) similar to the pattern of protein staining (Figure 1B, left panel). In addition, we could not detect any Ca^{2+}-independent CaM-binding proteins in the eluate (Figure 1B, right panel). These results suggest that we purified a comprehensive set of Ca^{2+}/CaM-binding proteins from rat brain. Next, to identify the CaM-interacting proteins, we excised 20 gel slices in the range from ~30 to >200 kDa from the SDS−PAGE gel in which the eluate from CaM−GST-coupled glutathione-Sepharose had been separated. The slices were then subjected to in-gel digestion with trypsin. The

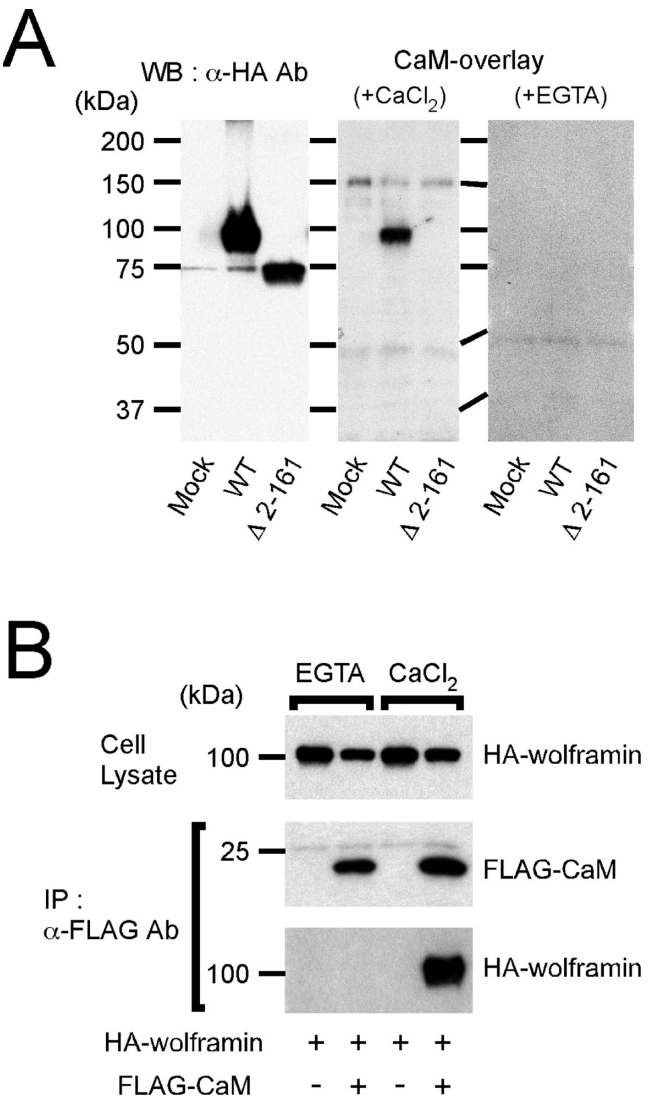

FIGURE 2: CaM binding of rat wolframin *in vitro* and in transfected cells. (A) COS-7 cells were transfected with 10 μg of an empty plasmid (Mock) or with 10 μg of HA-rat wolframin wild-type (WT) or a mutant lacking residues 2−161 (Δ2−161) as an expression vector. After being cultured for 40 h, membrane fractions were prepared and then subjected to Western blot analysis using an anti-HA antibody (left panel) or CaM overlay analysis in the presence of 1 mM $CaCl_2$ (center panel) or 2 mM EGTA (right panel) as described in Experimental Procedures. (B) COS-7 cells were transfected with 5 μg of HA-rat wolframin wild-type expression vector together with (+) or without (−) 5 μg of FLAG-CaM expression vector. After the samples had been cultured for 40 h, FLAG-CaM was immunoprecipitated with an anti-FLAG antibody, and then immunoprecipitates were subjected to Western blot analysis using either an anti-FLAG antibody (center panel) or an anti-HA antibody (bottom panel). Cell lysate from transfected COS-7 cells was analyzed by Western blot with an anti-HA antibody (top panel). Results were representative of at least three independent experiments.

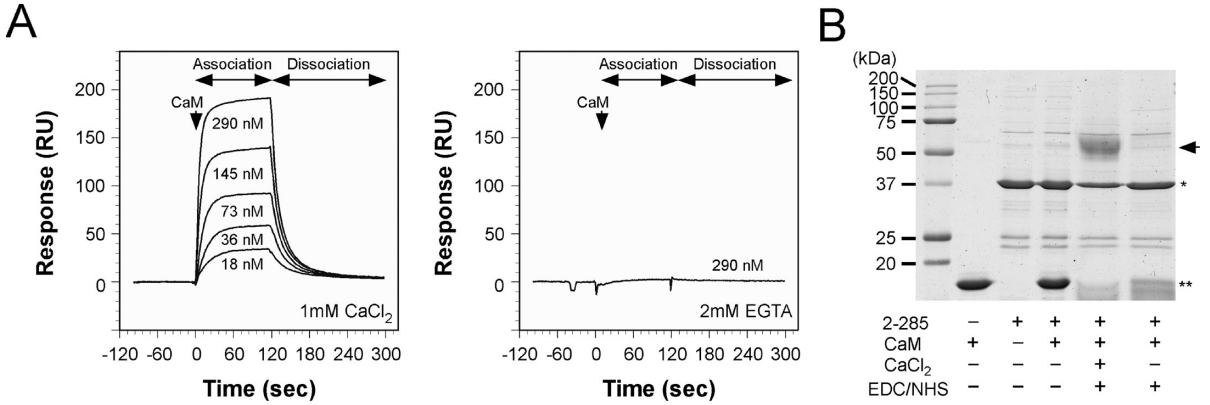

FIGURE 3: Characterization of CaM binding of the rat wolframin cytoplasmic domain. (A) Family of SPR traces showing steady-state binding responses of the cytoplasmic domain (residues 2−285) of rat wolframin (1625 RU immobilized) to 18−290 nM CaM in buffer containing 1 mM $CaCl_2$ (left panel) or 2 mM EGTA (right panel; a binding response with 290 nM CaM is shown) as described in Experimental Procedures. Nonspecific binding responses to the sensor chip surface have been subtracted. (B) Zero-length cross-linking of the rat wolframin cytoplasmic domain with CaM. CaM was incubated for 15 min at 25 °C with (+) or without (−) 50 mM NHS and 30 mM EDC (EDC/NHS) in 150 mM NaCl, 25 mM HEPES (pH 7.5), and 0.2 mM $CaCl_2$. The activation step was terminated by addition of 2-mercaptoethanol, and purified cytoplasmic domain (residues 2−285) of rat wolframin 2−285 was added (+) in the presence of 1 mM $CaCl_2$ ($CaCl_2$ +) or 4 mM EGTA ($CaCl_2$ −). Protein solutions were incubated for 1 h at 25 °C, and the products were analyzed by SDS−12.5% PAGE. The asterisk and double asterisk indicate the cytoplasmic domain (residues 2−285) of rat wolframin and CaM, respectively. An arrow indicates a cross-linked product. The left lane in panel B shows the molecular mass markers. Similar results were obtained for at least three independent experiments.

digested peptides eluted from each slice were subjected to LC−MS/MS analysis to identify the proteins by searching the NCBI database (see Supplemental Data 1 of the Supporting Information). Among the proteins we detected from CaM−GST-coupled glutathione-Sepharose samples, 36 known CaM-binding proteins were identified (Table 1). These CaM-binding proteins include CaM-regulated enzymes such as protein kinases (CaM kinases I, II, and IV, CaM-K kinases, and phosphorylase kinase), phosphatase (calcineurin), phosphodiesterase, nNOS, phosphofructokinase, and cytoskeletal proteins such as MARCKS, spectrin, tubulin, adducin, and synapsins as well as membrane proteins such as the IP_3 receptor and Ca^{2+}-ATPase. Therefore, this approach might provide efficient and comprehensive identification of CaM targets.

Identification of Wolframin from Rat Brain as a CaM-Binding Protein. In addition to identifying known targets for CaM with this functional proteomic approach, we obtained three peptides (T-1, SAGEATTPEPR; T-2, QLSSETDLER; T-3, GHEEDDITK) in the eluate that completely matched the corresponding amino acid sequence of rat wolframin (see Supplemental Data 2 of the Supporting Information and Figure 4C). Wolframin is the endoplasmic reticulum (ER) membrane protein (*30*) encoded by the Wolfram syndrome gene (*WFS1*), which has 890 amino acid residues and an apparent molecular mass of ~100 kDa (*22*) and has not previously been shown to interact with calmodulin. Mutations of the human *WFS1* gene in the coding region are responsible for Wolfram syndrome, which is an inherited association of juvenile-onset insulin-dependent diabetes mellitus, optic atrophy, and further neurological and endocrinological abnormalities (*24*,*25*,*31*−*33*); however, the molecular function of the protein remains unclear. Ca^{2+}-dependent interaction of rat wolframin with CaM was confirmed by a CaM overlay and an immunoprecipitation assay (Figure 2). We prepared and analyzed membrane fractions from transfected COS-7 cells. As shown in Figure 2A, HA-tagged rat wolframin expressed in COS-7 cells gives a CaM binding signal at a position of ~90 kDa in the presence of 1 mM $CaCl_2$ (Figure 2A, center panel), but CaM binding was not detected in the absence of Ca^{2+} (Figure 2A, right panel). When we deleted a part of the N-terminal cytoplasmic domain (residues 2−161) (*22*), the deletion mutant of wolframin no longer bound to the Ca^{2+}/CaM complex, indicating that the Ca^{2+}/CaM-binding region is apparently located in the N-terminal cytoplasmic domain. Next we tested whether wolframin is capable of binding to Ca^{2+}/CaM in intact cells by immunoprecipitation (Figure 2B). HA-tagged rat wolframin was expressed with or without FLAG-tagged CaM in COS-7 cells, and then FLAG-CaM was immunoprecipitated from the transfected cell lysate by using anti-FLAG antibody in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. Western blot analysis with the anti-HA antibody showed that the HA-tagged rat wolframin was specifically co-immunoprecipitated with FLAG-CaM in the presence of $CaCl_2$, but HA-wolframin−FLAG-CaM interaction was not detected in the absence of Ca^{2+} (Figure 2B, bottom panel). These results indicated that wolframin is capable of binding the Ca^{2+}/CaM complex in the cells as well as *in vitro*.

Characterization of CaM Binding of the Rat Wolframin Cytoplasmic Domain. Next we characterized CaM binding of rat wolframin by surface plasmon resonance (SPR) analysis and zero-length cross-linkng (Figure 3). Wolframin is an ER membrane protein with nine predicted transmembrane segments (*30*); thus, it is difficult to prepare recombinant full-length wolframin, and the N-terminal region has shown to be involved in CaM binding of wolframin (Figure 2A). Therefore, the N-terminal cytoplasmic domain of wolframin (residues 2−285) was immobilized on an SPR sensor chip (CM5) to examine its CaM binding ability. Figure 3A shows the binding responses of the cytoplasmic domain (residues 2−285) of rat wolframin to injections of a series of CaM concentrations with 1 mM $CaCl_2$ (left panel) and 2 mM EGTA

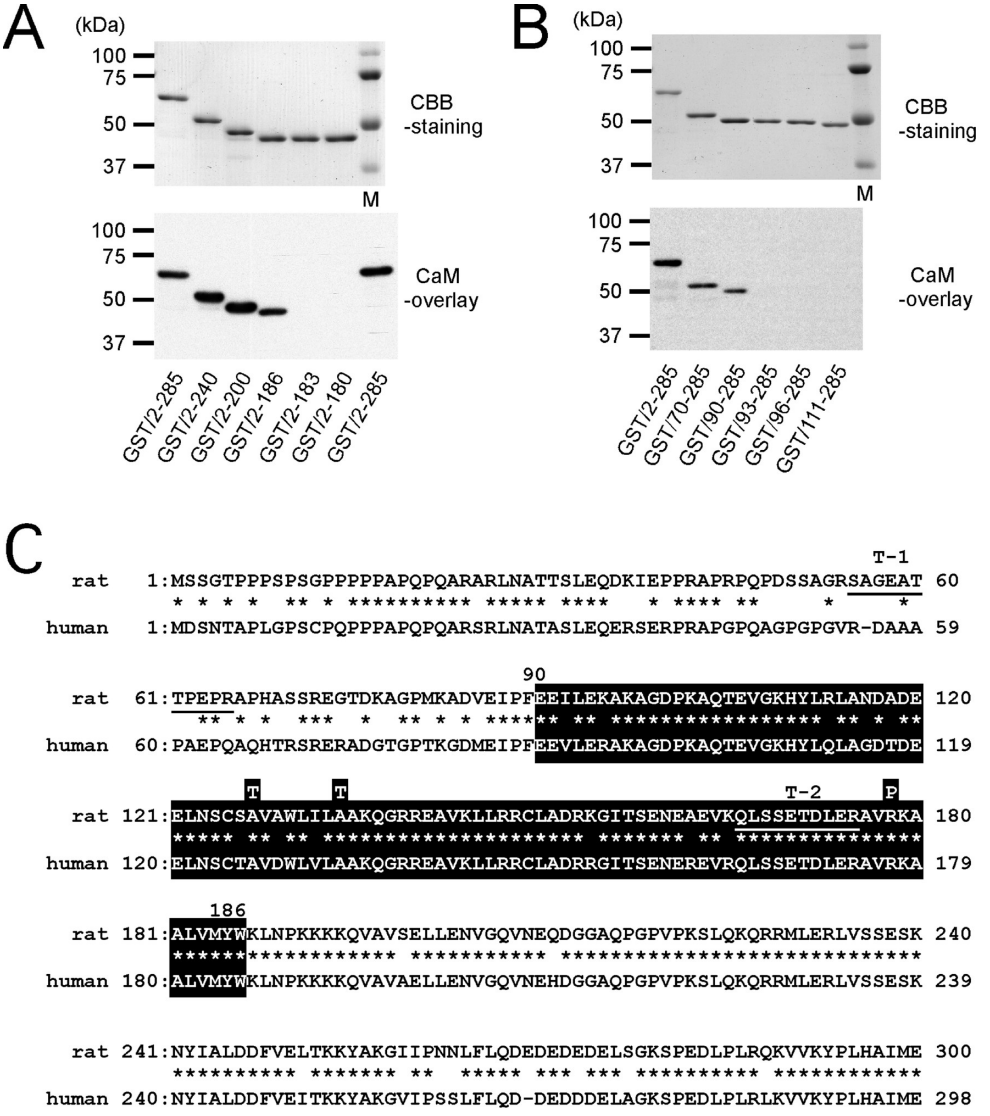

FIGURE 4: Identification of the CaM-binding region in rat wolframin. (A) Truncation mutants of the GST−rat wolframin cytoplasmic domain proteins (residues 2−285, 2−240, 2−200, 2−186, 2−183, or 2−180) (~1 μg) were purified and subjected to SDS−PAGE followed by protein staining (top panel) or CaM overlay analysis in the presence of 1 mM $CaCl_2$ (bottom panel). Lane M in the top panel shows the molecular mass markers. (B) Deletion mutants of the GST−rat wolframin cytoplasmic domain proteins (residues 2−285, 70−285, 90−285, 93−285, 96−285, or 111−285) (~1 μg) were prepared and subjected to SDS−PAGE followed by protein staining (top panel) or CaM overlay analysis in the presence of 1 mM $CaCl_2$ (bottom panel). Lane M in the top panel shows the molecular mass markers. (C) Amino acid sequences for the N-terminus of rat and human wolframin as deduced from cDNAs (GenBank accession number AF136378 and NM_006005). Identical amino acid residues between rat and human wolframin are denoted with asterisks. The amino acid sequences of the peptides (T-1 and T-2, underlined) from rat wolframin, eluted from CaM−GST-coupled glutathione-Sepharose, were determined by LC−MS/MS as shown in Figure 1. The CaM-binding region (residues 90−186) of wolframin determined in this study is indicated with a box. Three mutations [Ala126(127 in rat protein)Thr (*46*), Ala133(134 in rat protein)Thr (*44*), and Arg177(178 in rat protein)Pro (*41*)] associated with Wolfram syndrome are indicated.

(right panel). Nonspecific binding responses to the sensor chip surface have been subtracted out (see Experimental Procedures). CaM bound to the cytoplasmic domain (residues 2−285) of rat wolframin in a concentration-dependent manner, with rapid kinetics. Binding of CaM to the cytoplasmic domain (residues 2−285) was completely calcium-dependent, with a calculated K_D for CaM of 0.15 μM. To analyze the stoichiometry of CaM binding to wolframin, we used a two-step zero-length cross-linking method using EDC in the presence of NHS (*29*). While the apparent molecular mass of the cytoplasmic domain (residues 2−285) of rat wolframin on SDS−PAGE is 37 kDa, the molecular mass of the cross-linked product in the presence of Ca^{2+} and CaM (~17 kDa) was increased to 55 kDa (Figure 3B). However, in the absence of Ca^{2+}, cross-linked products were not observed (Figure 3B, right lane). These observations clearly suggest that wolframin bound to an equimolar amount of CaM in a Ca^{2+}-dependent manner.

Identification of the CaM-Binding Region in Rat Wolframin. To precisely map the Ca^{2+}/CaM-binding site in wolframin, we analyzed a GST-fused cytoplasmic domain (residues 2−285) of rat wolframin, including various N-terminal deletion and C-terminal truncation mutants by CaM overlay in the presence of 1 mM $CaCl_2$. In good agreement with results from the SPR and cross-linking analysis shown in panels A and B of Figure 3, CaM overlay also showed that the GST-fused N-terminal domain (residues 2−285) of wolframin is capable of binding with Ca^{2+}/CaM (Figure 4A,B). Truncation at

residue 186 (GST/2−186) preserves the protein's CaM binding ability, but truncation of a further three amino acid residues (GST/2−183) completely abolished CaM binding (Figure 4A). A deletion mutant at residue 90 (GST/90−285) bound Ca^{2+}/CaM in a manner similar to that of GST/2−285 or GST/70−285, but a further three-amino acid residue deletion mutant (GST/93−285) failed to bind Ca^{2+}/CaM (Figure 4B). These results indicate that the minimum Ca^{2+}/CaM-binding region of rat wolframin is located from Glu90 to Trp186 (Figure 4C). As compared to well-characterized CaM-binding peptides derived from various CaM-Ks that are amphiphatic α-helices composed of 20−26 amino acid residues (*34*−*37*), the CaM-binding region in wolframin identified in this study is apparently distinct from the conventional CaM-binding motif.

Effect of Missense Mutations Associated with Wolfram Syndrome on CaM Binding of Wolframin. Wolfram syndrome is caused by homozygous mutations (*24*, *25*) as well as compound heterozygous mutations in *WFS1* (*38*−*41*). To date, ~130 distinct mutations in *WFS1* have been identified in Wolfram syndrome individuals, including a variety of missense, nonsense, and frameshift insertion/deletion mutations (*24*, *25*, *39*, *40*, *42*−*46*). Although these mutations are distributed along the entire gene without any apparent mutational hot spot, the amino acid residues of three known mutations [Ala126(127 in rat protein)Thr (*46*), Ala133(134 in rat protein)Thr (*44*), and Arg177(178 in rat protein)Pro (*41*)] in human wolframin are conserved in the rat counterpart and have been shown to be located in the CaM-binding region identified in this study (see Figure 4C). Therefore, we examined whether these mutations affect the CaM binding ability of wolframin using HA-tagged full-length wolframin expressed in COS-7 cells and a GST−wolframin cytoplasmic fragment (residues 2−285) (Figure 5). Interestingly, CaM overlay analysis revealed that all three of the single mutations (Ala127Thr, Ala134Thr, and Arg178Pro) completely abolished the CaM binding ability of HA-wolframin (Figure 5A). This result was confirmed by an experiment using the GST−wolframin cytoplasmic fragment (residues 2−285), including the wild-type and three mutant fusion proteins (Figure 5B). These results indicate that the impairment of CaM binding may cause loss of function of wolframin, resulting in its association with Wolfram syndrome.

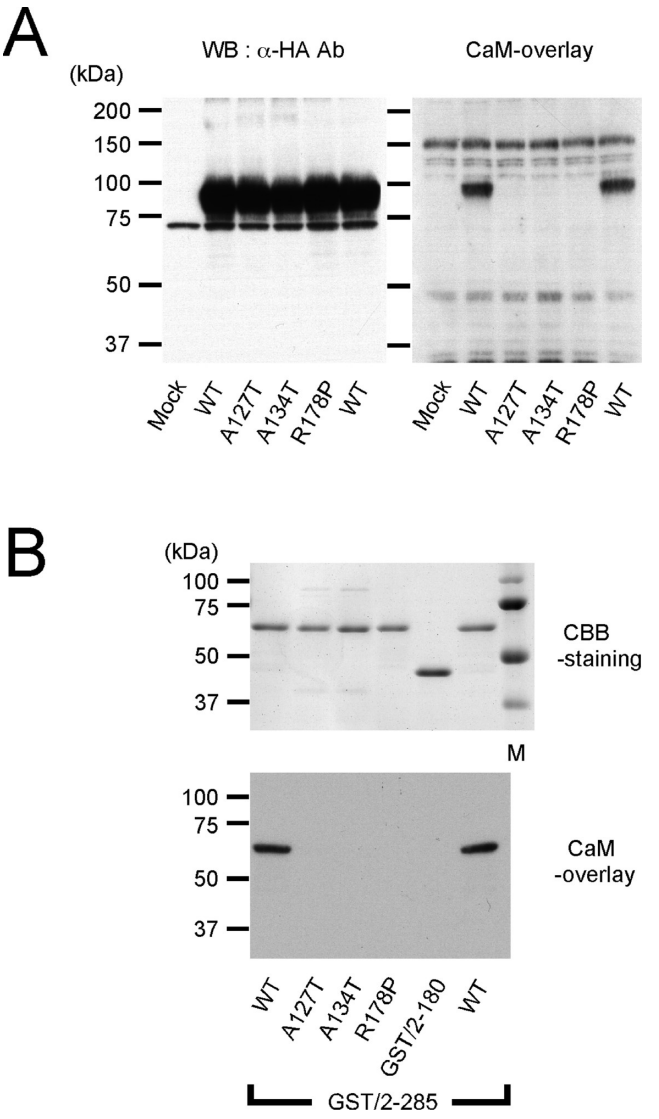

FIGURE 5: Impairment of the CaM binding ability of rat wolframin by mutations associated with Wolfram syndrome. (A) COS-7 cells were transfected with 10 μg of an empty plasmid (Mock) or with 10 μg of HA-rat wolframin wild-type or mutant (Ala127Thr, Ala134Thr, and Arg178Pro) expression vector. After being cultured for 40 h, membrane fractions were prepared and then subjected to Western blot analysis using an anti-HA antibody (left panel) or CaM overlay analysis in the presence of 1 mM $CaCl_2$ (right panel) as described in Experimental Procedures. (B) GST−rat wolframin cytoplasmic domain (residues 2−285) of the wild-type or mutants (Ala127Thr, Ala134Thr, and Arg178Pro) (~1 μg) was purified and subjected to SDS−PAGE followed by protein staining (top panel) or CaM overlay analysis in the presence of 1 mM $CaCl_2$ (bottom panel). Lane M in the top panel shows the molecular mass markers. WT, wild-type; A127T, Ala127Thr; A134T, Ala134Thr; R178P, Arg178Pro. Similar results were obtained for at least three independent experiments.

DISCUSSION

In this report, we describe the development of a proteomic approach to identifying potential CaM targets in an effort to discover novel Ca^{2+}/CaM-mediated signaling pathways. We have attempted to improve the conventional single-step purification of CaM-binding proteins by using recombinant GST-fused CaM as an affinity ligand followed by trapping of the ligand/CaM-binding protein complex with glutathione-Sepharose instead of CaM-coupled Sepharose. After the complex had been trapped with glutathione-Sepharose, the EGTA eluate was analyzed using LC−MS/MS to identify CaM targets. On the basis of this method, we have identified 36 known CaM-binding proteins, including CaM-regulated enzymes, cytoskeletal proteins, and membrane proteins, indicating that the functional proteomic approach is reasonably efficient for comprehensive analysis of CaM targets. Therefore, this method could be applied in searching target molecules for other Ca^{2+}-binding proteins such as S100 family or neuronal Ca^{2+}-sensor proteins as well as for CaM targets in various tissues.

In addition to detection of these known targets for CaM, wolframin, the product of the Wolfram syndrome gene (*WFS1*), has been shown to interact with CaM in a Ca^{2+}-dependent manner with rapid kinetics. We confirmed

Ca^{2+}/CaM binding of wolframin in transfected cells according to the results which showed that HA-tagged wolframin was co-immunoprecipitated with coexpressed FLAG-tagged CaM in a Ca^{2+}-dependent manner (Figure 2B). However, we cannot rule out the possibility that CaM and wolframin could interact following homogenization of the cells. More direct proof of *in vivo* interaction of the two molecules will require further analyses. Wolframin is an N-glycosylated ER membrane protein that is composed of an N-terminal cytoplasmic domain, nine predicted transmembrane segments, and a C-terminal luminal domain (*22*, *30*). We determined that the CaM-binding region at the N-terminal cytoplasmic domain (Glu90−Trp186) in rat wolframin is apparently very long compared to known CaM-binding sequences, including CaM-KII (1−10 motif) (*36*), MLCK (1−14 motif) (*34*, *35*), and CaM-K kinase (1−16 motif) (*37*). Since we observed stoichiometric binding (1:1) of CaM to wolframin, the N-terminal CaM-binding region (Glu90−Trp186) likely forms a compact conformation to bind Ca^{2+}/CaM. Therefore, it is of interest to know the structure of the Ca^{2+}/CaM−wolframin complex.

Previous studies demonstrated that mutations in the *WFS1* gene are associated with a severe disorder in humans, the Wolfram syndrome, which is characterized by juvenile non-autoimmune diabetes mellitus and optic atrophy. Numerous disease-causing mutations in the *WFS1* gene comprising loss-of-function mutations such as stop, frameshift, splice site, and missense mutations have been identified. However, the molecular function of the *WFS1* gene product (wolframin) has not been elucidated; thus, it is difficult to assess or predict the effect of these mutations on the biological function of wolframin. Therefore, this is the first direct demonstration indicating that one of the biochemical functions of wolframin, namely CaM binding, is impaired by multiple distinct mutations that are associated with Wolfram syndrome. These findings strongly suggest that CaM binding could be important in regulating wolframin function, and therefore, wolframin may participate in a novel Ca^{2+} signal transduction system.

Channel activity of microsomal membranes from wolframin-expressing *Xenopus* oocytes has been observed, and the level of cytosolic Ca^{2+} in the oocytes was increased by overexpression of wolframin (*47*). This observation suggested that wolframin is associated with channel activity in the ER membrane as a novel ER Ca^{2+} channel or as a regulator of ER Ca^{2+} channel activity, which is involved in the regulation of intracellular Ca^{2+} homeostasis. Furthermore, a recent study using *WFS1*-knockdown and -overexpressing HEK293 cells has shown that wolframin positively modulates ER Ca^{2+} levels by increasing the rate of Ca^{2+} uptake (*48*), although the molecular details of this regulation are still unknown. These two observations have suggested a linkage of wolframin with Ca^{2+} signaling that is strongly supported by our results which show that wolframin is a target of Ca^{2+}/CaM. With the results of our study, CaM might be a sensor of cytosolic Ca^{2+} concentration for regulating wolframin function, such as Ca^{2+} channel activity or modulating the filling state of the ER Ca^{2+} store. Once this regulatory mechanism is impaired by mutation, the loss of function of wolframin may cause the disease phenotype. It is noteworthy that channel activity of another ER Ca^{2+} channel, the IP_3 receptor, is also regulated by CaM (*49*) or CaM-related Ca^{2+}-binding protein (CIB1) (*50*).

In conclusion, we have identified a novel interaction between the ubiquitously expressed Ca^{2+}-binding protein CaM and the product of the Wolfram syndrome gene (*WFS1*), wolframin. This finding may shed light on the dynamic regulation of the wolframin pathway by means of increasing the cytosolic Ca^{2+} concentration in various cells, resulting in control of intracellular Ca^{2+} homeostasis as well as molecular details of the disease. Future studies specifically designed to explore molecular function and regulation of wolframin by the Ca^{2+}/CaM complex will be necessary to understand the physiological significance of the Ca^{2+}/CaM−wolframin pathway as well as the molecular mechanism for the pathology of Wolfram syndrome.

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SUPPORTING INFORMATION AVAILABLE

Supplemental Data 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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