Structural Basis for the Golgi Association by the Pleckstrin Homology Domain of the Ceramide Trafficking Protein (CERT) *

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Background: The CERT PH domain is indispensable for the ER-to-Golgi ceramide transport.
Results: The three-dimensional structure and interaction study revealed the Golgi recognition mode of the CERT PH domain.
Conclusion: The basic groove in the CERT PH domain plays a critical role in the Golgi recognition.
Significance: Conservation of the basic groove within lipid transporters uncovers functional significance of the structural motif.

Ceramide transport from the endoplasmic reticulum to the Golgi apparatus is crucial in sphingolipid biosynthesis, and the process relies on the ceramide trafficking protein (CERT), which contains pleckstrin homology (PH) and StAR-related lipid transfer domains. The CERT PH domain specifically recognizes phosphatidylinositol 4-monophosphate (PtdIns(4)P), a characteristic phosphoinositide in the Golgi membrane, and is indispensable for the endoplasmic reticulum-to-Golgi transport of ceramide by CERT. In this study, we determined the three-dimensional structure of the CERT PH domain by using solution NMR techniques. The structure revealed the presence of a characteristic basic groove near the canonical PtdIns(4)P recognition site. An extensive interaction study using NMR and other biophysical techniques revealed that the basic groove coordinates the CERT PH domain for efficient PtdIns(4)P recognition and localization in the Golgi apparatus. The notion was also supported by Golgi mislocalization of the CERT mutants in living cells. The distinctive binding modes reflect the functions of PH domains, as the basic groove is conserved only in the PH domains involved with the PtdIns(4)P-dependent lipid transport activity but not in those with the signal transduction activity.

Sphingolipids are the major structural element in eukaryotic membranes, and they function as crucial signal mediators in a variety of important biological processes, including apoptosis, inflammation, etc. (1). Sphingolipid synthesis involves multiple steps of metabolic conversion accomplished by a series of enzymes localized in the ER,3 the Golgi apparatus, and the plasma membrane. Therefore, the transportation of sphingolipids from one organelle to others must be highly organized. The interorganellar trafficking of sphingolipids is mediated in either a vesicular or nonvesicular manner (2). For instance, in mammalian cells, a de novo synthesized ceramide in ER membranes is transported to the Golgi in a nonvesicular manner by an ER-to-Golgi specific ceramide transporter (CERT, also known as GPBP2A26, a splicing variant of Goodpasture antigen-binding protein) (3, 4). The functional impairment of CERT results in the complete loss of the ceramide trafficking activity in cells, indicating its essential role in sphingolipid biogenesis (4).

CERT is a cytoplasmic 68-kDa protein, and it contains two distinct functional domains as follow: the N-terminal pleckstrin homology (PH) domain (~100 amino acid residues) and the C-terminal StAR-related lipid transfer (START) domain (~230 amino acid residues) (Fig. 1A) (4). The middle region between the PH and START domains is not predicted to form a globular fold; however, it contains a short peptide motif with the consensus sequence EFFDAE, named the “two phenylalanines in an acidic trait” (FFAT) motif (Fig. 1A) (5). The FFAT motif interacts with VAMP-associated protein, an ER-resident type II membrane protein, and the interaction is considered to be crucial for association of CERT with the ER membrane (5).

The CERT START domain specifically recognizes and then extracts ceramide from lipid membranes (4). This domain is

3 The abbreviations used are: ER, endoplasmic reticulum; CERT, ceramide trafficking protein; PH, pleckstrin homology; START, StAR-related lipid transfer; PtdIns(4)P, phosphatidylinositol 4-monophosphate; TCS, transferred cross-saturation; SPR, surface plasmon resonance; CSP, chemical shift perturbation; diC6, deuteranoyl.
essential for the activities, as the deletion of the START domain impairs the CERT ceramide transfer activity (4). Recently, the three-dimensional structure of the CERT START domain was determined by x-ray crystallography (6), and the molecular mechanisms for its membrane interaction, and subsequent ceramide extraction, were revealed (7, 8).

Interestingly, however, the CERT START domain alone is not sufficient for the ER-to-Golgi ceramide transport in vivo (4, 5). Indeed, the PH and START domains are both indispensable for the CERT ceramide transport activity, as the impairment of the CERT PH domain also causes the complete loss of the ceramide transport activity (4, 5).

The CERT PH domain specifically recognizes PtdIns(4)P in membranes (4). Because PtdIns(4)P is the most abundant and preferentially distributed phosphoinositide in trans-Golgi membranes, it is assumed that the PH domain is required for efficient association of CERT with the Golgi membrane (4, 5). At present, however, the detailed mechanism, by which the CERT PH domain interacts with PtdIns(4)P in the Golgi membrane to exert the transportation activity, remains unclear.

In this study, we determined the three-dimensional structure of the CERT PH domain by using solution-state NMR techniques. The structure revealed that the CERT PH domain has a characteristic basic groove near the PtdIns(4)P recognition site. Extensive interaction studies using NMR and other biochemical methods indicated that the recognition of the PtdIns(4)P headgroup by the CERT PH domain is weak, but the interaction with PtdIns(4)P in the Golgi membrane is enhanced by an electrostatic interaction between the basic groove and the phospholipid membrane. The Golgi mislocalization of the CERT mutants in living cells supported the functional importance of the CERT PH domain (4, 5). Indeed, the PH and START domains are both indispensable for its membrane interaction, and subsequent ceramide extraction, were revealed (7, 8).

EXPERIMENTAL PROCEDURES

All chemicals were purchased from Nacalai Tesque, Inc., unless specifically noted. The diC2-PtdIns(4)P and inositol (1,4)-bisphosphate were purchased from Echelon Biosciences, Inc.

Expression and Purification of the CERT PH Domain—The CDNA containing human CERT was prepared as described previously (4). The cDNA fragment encoding amino acid residues 23–117 from the full-length CERT was amplified by PCR and cloned into the pET28a(+)/H11001 plasmid (Novagen). The plasmid was transformed into the Escherichia coli BL21(DE3) strain. The uniformly $^{15}$N- and $^{13}$C/$^{15}$N-labeled CERT PH domains were prepared by growing the transformants in M9 media, containing 0.1% (w/v) $[^{15}$N]ammonium chloride (99 atom % $^{15}$N) and either 0.2% (w/v) D-glucose or D-$[^{13}$C$_6$]glucose (98 atom % $^{13}$C), respectively. Similarly, the uniformly $^2$H/$^{15}$N-labeled protein was prepared by using M9 medium, containing 100% D$_2$O (99.8 atom % $^2$H), 0.1% (w/v) $[^{15}$N]ammonium chloride, and 0.2% (w/v) D-$[^{13}$H$_7$,$^{13}$C$_5$]glucose (both 98 atom % $^2$H and $^{13}$C). The transformants were grown at 37 °C to an $A_{600}$ of 0.6–0.8, and protein expression was induced with 0.5 mm isopropyl 1-thio-β-D-galactopyranoside. The cultivation was continued for an additional 16 h at 25 °C, and the cells were harvested by centrifugation.

The cell pellets were resuspended in lysis buffer, containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 2.5 mM 2-mercaptoethanol. The suspended cells were lysed by sonication and subjected to centrifugation. The His$_6$-tagged CERT PH domain was purified from the supernatant of the centrifuged lysate by metal-chelating chromatography, using His-Select resin and the tobacco etch virus protease (Invitrogen). The digest was passed through His-Select resin and the tobacco etch virus protease. The CERT PH domain was further purified by anion exchange column chromatography, using a Mono Q ion exchange column (GE Healthcare).

The recombinant CERT PH domain consists of 96 amino acids, corresponding to Val-24 to Thr-117 in the full-length CERT protein and two glycine residues at the N terminus, which remained after the digestion of the N-terminal purification tag. Site-directed alanine-scanning mutagenesis of the CERT PH domain was performed by QuikChange mutagenesis (Stratagene), according to the manufacturer’s protocol. All mutants were expressed and purified in the same manner as the wild type.

Preparation of Liposomes—The phospholipids used in this study (1-palmitoyl-2-oleoylphosphocholine, 1-palmitoyl-2-oleoylphosphoethanolamine, 1-palmitoyl-2-oleoylphosphoserine, and D-myophosphatidylinositol 4-monophosphate (PtdIns(4)P)) were purchased from Avanti Polar Lipids or Echelon. For the PtdIns(4)P-embedded liposomes, 1-palmitoyl-2-oleoylphosphocholine/1-palmitoyl-2-oleoylphosphoethanolamine/1-palmitoyl-2-oleoylphosphoserine/PtdIns(4)P = 66:24:4:2.5:0.0% (molar ratio) were used. This phospholipid composition used here is similar to that in the Golgi membrane (9), and hereafter, we refer to the corresponding liposome as the “PtdIns(4)P-embedded Golgi-mimetic liposomes.” For the PtdIns(4)P-free liposomes, PtdIns(4)P was exchanged with 1-palmitoyl-2-oleoylphosphocholine. The phospholipid mixture was dissolved in chloroform/methanol (1:1 (v/v)) and was dried under a nitrogen flow to form a thin film. The lipid film was suspended by vortexing with a buffer (10 mM HEPES-NaOH (pH 7.2), 100 mM NaCl, and 5 mM tris(2-carboxyethylphosphine-HCl). Small unilamellar vesicles were prepared by using a Mini-Extruder (Avanti Polar Lipids, Inc.), as described previously (8).

NMR Spectroscopy—All NMR experiments were performed on a Bruker Avance 800 spectrometer equipped with a TXI cryogenic probe. All NMR spectra were recorded at 25 °C, using 0.10–0.25 mM CERT PH domain in 10 mM HEPES-NaOH buffer (pH 7.2), containing 100 mM NaCl, 5 mM tris(2-carboxyethylphosphine-HCl), and 7% D$_2$O. For recording two-dimensional $^1$H/$^1$H NOESY, two-dimensional $^1$H/$^{13}$C HSQC,
two-dimensional (HB)CB(CGCD)HD, two-dimensional (HB)CB(CGCDCE)HE, three-dimensional $^{13}$C-edited NOESY-HSQC, and three-dimensional HCCH-TOCSY spectra, 100% D$_2$O buffer was used instead. Site-specific resonance assignments of $^1$H, $^{15}$N, $^{13}$C nuclei of the CERT PH domain were achieved by a standard set of multidimensional triple resonance experiments, such as two-dimensional $^1$H-$^{15}$N HSQC, two-dimensional $^1$H-$^{13}$C CT-HSQC, three-dimensional HNCA CB, three-dimensional HN(CC)CACB, three-dimensional HN(CO)CACB, three-dimensional HN(CA)CO, three-dimensional $^{15}$N-edited NOESY-HSQC, four-dimensional $^{13}$C-$^{15}$N-edited NOESY, and three-dimensional MQ-(H)CCmHm-TOSCY (10–12). All NMR spectra were processed by TopSpin 2.1 (Bruker) or NMRPipe (13) and were analyzed with Sparky (Goddard and Kneller, SPARKY 3-NMR Assignment and Integration Software, University of California, San Francisco). The chemical shift assignments of the CERT PH domain were deposited in the Biological Magnetic Resonance Data Bank under accession number 11473.

In the chemical shift perturbation studies, 0.1 mM of uniformly $^{15}$N-labeled CERT PH domain was titrated with increasing concentrations (0.2–1.6 mM) of diC$_4$-PtdIns(4)P or Ins(1,4)P$_2$. The averaged chemical shift changes ($\Delta \delta$) in the two-dimensional $^1$H-$^{15}$N HSQC spectra were calculated with the equation $\Delta \delta = (\Delta \delta_{HI} + (\Delta \delta_{HN}/5))^{1/2}$, where $\Delta \delta_{HI}$ and $\Delta \delta_{HN}$ are the chemical shift changes of the amide protons and $^{15}$N nuclei in parts/million.

In the transferred cross-saturation (TCS) experiments (14, 15), the PtdIns(4)P-embedded Golgi-mimetic liposomes were mixed with the 0.20 mM of uniformly $^2$H-$^{15}$N-labeled CERT PH domain. The total lipid concentration in the sample was set to 1.6 mM. The proton content of the solvent was set to 20% (v/v), to suppress the intramolecular spin diffusion in the CERT PH domain (14–17). Saturation of the lipid signals was achieved by a series of Gaussian-shaped pulses with a 3-ms duration and a 1-ms delay between pulses (13, 18, 19). The center of the irradiation frequency was set to 3.2 ppm, which corresponds to the chemical shifts of the lipid headgroups (18). A control spectrum with the irradiation frequency set to $-10$ ppm was acquired in an interleaved manner. The irradiation time and the pre-scan delay were set to 0.35 and 2.65 s, respectively. The irradiation scheme saturates all of the aliphatic resonances from the phospholipids. The TCS experiment was also performed without liposomes, to estimate the effects of unwanted direct saturations to the protein. In the analyses, the signal intensity reductions ratios obtained with liposomes were normalized by those without liposomes.

**Structure Calculation**—Proton-proton distance restraints were obtained from two-dimensional $^1$H-$^1$H NOESY (mixing time, 100 ms), three-dimensional $^{13}$C-edited NOESY-HSQC (mixing time, 150 ms), and three-dimensional $^{15}$N-edited NOESY-HSQC (mixing time, 120 ms) spectra, using the CANDid algorithm of CYANA 2.1 (21). The backbone dihedral angle restraints (58 for $\phi$ and 56 for $\psi$ angles) were obtained from the program TALOS, using the $^1$H$_{\alpha}$, $^{15}$N$_{\alpha}$, $^{13}$C$_{\alpha}$, $^{13}$C$_{\beta}$, $^{13}$C$_{\gamma}$, and $^1$H chemical shifts (22). Distance restraints for 32 hydrogen bonds were introduced for sites exhibiting a slow amide proton exchange and an evident secondary structure, in a preliminary structure calculation without hydrogen bond information.

Using the distance and angular restraints obtained from the NMR experiments, structure calculations were performed with a simulated annealing protocol, using the program CYANA 2.1 (21). The 20 final structures with the lowest statistical violations to the experimental data were adopted from 100 independent calculations (23). The ensemble of the 20 structures was further refined by the program CNS with explicit solvent and electrostatics (24). The quality of the 20 final structures was evaluated using the program PROCHECK NMR (25). All structure figures were prepared using the program MOLMOL (26). The structure coordinates have been deposited in the Protein Data Bank, with the accession code 2RSG.

**Surface Plasmon Resonance (SPR) Experiments**—SPR measurements were performed using a Biacore 2000 instrument (GE Healthcare). The liposomes were immobilized on a sensor chip L1 (GE Healthcare), as described previously (8). The binding assay was performed in running buffer containing 10 mM HEPES-NaOH (pH 7.2), 100 mM NaCl, and 5 mM tris(2-carboxyethyl)phosphine-HCl at 25 °C. Various concentrations of the CERT PH domain (1–16 μM) were injected into the flow cells at a flow rate of 30 μl/min. The binding constants were obtained from the steady-state curve fitting of the SPR sensorsgrams, using the BIAevaluation 3.1 software (GE Healthcare).

**RESULTS**

**Characterization of the CERT PH Domain Binding to PtdIns(4)P-embedded Membranes**—To clarify the ligand specificities and affinities of the CERT PH domain, the binding constants between the CERT PH domain and phosphoinositide-embedded liposomes were measured by SPR methods. The dissociation constant ($K_d$) for the PtdIns(4)P-embedded Golgi-mimetic liposomes was $\sim 3.2 \mu M$ (Table 1), which is comparable with the previously reported value (27). Furthermore, the binding affinity for the PtdIns(4)P-embedded liposomes was $\sim 5$-fold higher than for the PtdIns(3)P- and PtdIns(5)P-embedded liposomes, and $\sim 5$-fold higher than for those embedded with PtdIns(4,5)P$_2$ (Table 1). These results support the proposal that the CERT PH domain specifically recognizes the PtdIns(4)P-containing lipid membrane, as reported previously (4, 27). Unexpectedly, the CERT PH domain showed weak but significant binding ($K_d = 220 \mu M$) to the PtdIns(4)P-free liposomes (Table 1). Although the binding was about 70-fold weaker, as compared with that of the PtdIns(4)P-containing liposomes, it

| Phosphoinositides | $K_d (\mu M)$ | Relative affinity
|-------------------|-------------|-----------------
| PtdIns(4)P        | 3.26 ± 0.23 | 1.00           |
| PtdIns(3)P        | 75.4 ± 33   | 0.05           |
| PtdIns(5)P        | 64.7 ± 33   | 0.06           |
| PtdIns(4,5)P$_2$  | 15.6 ± 1.9  | 0.21           |

$^a$ Phosphoinositides were embedded in liposomes as described under "Experimental Procedures."

$^b$ The $K_d$ values were measured by the SPR method, as described under "Experimental Procedures."

$^c$ Relative affinity, as compared with that of the PtdIns(4)P-embedded liposomes, was indicated.
suggested that the CERT PH domain also nonspecifically interacts with phospholipid membranes.

**Solution Structure of the CERT PH Domain**—To understand the structural basis of the Golgi-specific interaction, the solution structure of the CERT PH domain (residue 23–117) was determined, using standard multidimensional NMR techniques, as described under “Experimental Procedures.” The 20 lowest energy structures of the CERT PH domain were superimposed, as shown in Fig. 1A. The structural statistics of the lowest energy structures are shown in Table 2. The average pairwise root-mean-square differences of all secondary-structure elements indicated were 0.56 ± 0.09 Å for the backbone atoms and 1.20 ± 0.16 Å for all of the non-hydrogen atoms.

The CERT PH domain consists of seven β-strands (β1, 26–33; β2, 40–48; β3, 51–55; β4, 67–70; β5, 75–78; β6, 85–90; and β7, 93–98) and one C-terminal α-helix (αC, 106–115) (Fig. 1C). The main chain trace of the CERT PH domain falls in a regular PH domain fold (28, 29). The core structure forms a closely packed “β-sandwich,” with two nearly orthogonal antiparallel β-sheets (β1-β4 and β5-β7). The C-terminal α-helix lies on the top of the β-sandwich, and two loops connecting β1/β2 and β3/β4 are at the “exposed” end of the β-sandwich. The β1/β2 anti-parallel strands are almost twice as long as the β3/β4 anti-parallel strands. This feature makes half of the β1/β2 anti-parallel β-strands and the β1/β2 loop protrude from the core β-sandwich. The β1/β2, β3/β4, and β7/αC loops of the CERT PH domain showed higher deviations, as compared with the other elements. In the two-dimensional 1H-15N HSQC spectrum, four backbone amide cross-peaks from the β1/β2 loop (Asn-35, Tyr-36, Ile-37, and Gly-39) and one from the β7/αC loop (Asp-103) were invisible. This might be due to the presence of multiple conformations or solvent exchanges on an intermediate NMR time scale for these sites.

Fig. 1D shows the electrostatic potential surfaces of the CERT PH domain. On the surface of the CERT PH domain, the basic residues are clustered in the middle of the molecule, forming a basic groove around the protruding part of the β1/β2 region. The basic groove stretches from the “exposed end” to the “side surface” of the β-sandwich, and it includes seven basic residues (Lys-32, Arg-43, Lys-56, Arg-66, His-79 Arg-85, and Arg-98) (Fig. 1, C–E). Conversely, the protruding β1/β2 region that is surrounded by the basic groove mainly consists of aromatic and/or hydrophobic residues, such as Trp-33, Tyr-36, Ile-37, and Trp-40 (Fig. 1E).

**Determination of the PtdIns(4)P-binding Site on the CERT PH Domain**—To identify the PtdIns(4)P-binding site on the CERT PH domain, we performed chemical shift perturbation (CSP) experiments. Fig. 2A shows an overlay of two-dimensional 1H-15N HSQC spectra of the [U-15N]CERT PH domain with various concentrations of water-soluble diC4-PtdIns(4)P. As exemplified by the backbone amide signals from Trp-33 and Trp-40, as well as the side chain NH2 signals from Asn-35 (enlarged panels in Fig. 2A), several cross-peaks exhibited significant chemical shift changes upon the addition of diC4-PtdIns(4)P. Most of the residues exhibited typical “fast-exchanging” CSPs, which indicate rapid binding/unbinding events between the CERT PH domain and diC4-PtdIns(4)P. The affinity of diC4-PtdIns(4)P for the CERT PH domain was determined by a global fitting to the titration curves of six different amino acids, and the $K_D$ for diC4-PtdIns(4)P was 763 ± 13 μM (Fig. 2B). The normalized chemical shift changes caused by the addition of 16 eq (1.6 mM) of diC4-PtdIns(4)P were summarized in Fig. 2C, and the residues with significant CSPs were mapped onto the tertiary structure of the CERT PH domain (Fig. 2D). The residues on the β1/β2 loop, the C-terminal of the β3 strand, the C-terminal of the β3/β4 loop, as well as the middle of the β7 strand exhibited significant CSPs (Fig. 2C). The mapping of these residues clearly defined a phosphatidylinositol phosphate binding pocket in the CERT PH domain, indicating that the exposed end of the basic groove is the specific PtdIns(4)P-binding site (Fig. 2D). The titration of inositol (1,4)-bisphosphates further supported this notion (supplemental Fig. S1). The side chain of Arg-43 is located in the middle of the binding site (Fig. 2D). However, the side chain Hε-Ne resonance could not be observed in the two-dimensional 1H-15N HSQC spectrum of the CERT PH domain, probably due to the broadening induced by chemical exchange with the water resonance and/or fluctuation among multiple conformational states. Nevertheless, the mutation of the residue significantly reduced the binding affinity for the Golgi-mimetic liposomes, as discussed later (Fig. 4). It indicates that the PtdIns(4)P-binding pocket in the CERT PH domain is located in the common phosphatidylinositol phosphate-binding site of the other PH domains (28, 30). We also attempted to determine the tertiary structure of the CERT PH domain bound to diC4-PtdIns(4)P; however, it was not successful because no intermolecular NOE was observed possibly due to the low binding affinity between the two molecules.

The affinity of the CERT PH domain for the soluble form of PtdIns(4)P is quite weak, with a dissociation constant in the millimolar range (Fig. 2B), indicating that the exposed end of the basic groove is the specific PtdIns(4)P-binding site (Fig. 2D). Conversely, the affinity of the CERT PH domain for the PtdIns(4)P-embedded Golgi-mimetic liposomes measured by the SPR method was 3 μM (Table 1). This indicates the presence of additional interactions between the PtdIns(4)P-embedded membrane and the CERT PH domain.

**Determination of the Membrane-interacting Site on the CERT PH Domain**—To identify the additional membrane-interaction site in the CERT PH domain, we performed TCS experiments (14, 15). As outlined in Fig. 3A, the saturation of the phospholipid resonances in liposomes is transferred to the amide-proton resonances at a membrane interacting surface, which in turn is detected as the attenuation of the CERT PH domain amide signals. It is worth noting that the NMR signals of the bound-state CERT PH domain would not be detected due to the large molecular weight of the liposomes (over a few MDa). However, the rapid exchange between the free and bound states of the CERT PH domain allows us to detect the saturation caused in the bound state as the signal reduction in the free state (15, 17). Under the present experimental conditions, the two-dimensional 1H-15N HSQC resonances from the CERT PH domain were almost identical to those in the free state. The fraction of the bound-state CERT PH domain in the NMR sample is estimated to be less than 10%, and the dissociation rate constant between the CERT PH domain and the PtdIns(4)P-
Structure and Interaction of CERT PH Domain

A

N-terminus | PH | FFAT motif | START | C-terminus

Interaction | Interaction | Interaction

PtdIns(4)P | VAPs | Ceramide

B

C

D

Front-view | Right-view | Back-view

90° | 90°

E

Front-view | Back-view

180°

“Basic groove”
embedded Golgi-mimetic liposomes is sufficiently fast (~1.0 s⁻¹) (supplemental Fig. S2). The bound population and the dissociation rate constants are compatible conditions for a successful TCS experiment, as indicated in the recent theoretical analysis of the TCS method (16). Assuming a 1-μs rotational correlation time, a radio frequency irradiation of 0.3 s would cause >30% signal reductions for protons within 5 Å from the interface (16).

The irradiation of the Golgi-mimetic liposome resulted in specific intensity losses in the CERT PH domain resonances (Fig. 3, B and C). The residues that showed over 30% intensity reductions, as compared with the control spectrum, are colored and mapped on the three-dimensional structure of the CERT PH domain (Fig. 3D). Specifically, the protruding part of the β1/β2 sheet, including the β1/β2 loop (main chain HN resonances of Ser-31, Lys-32, Trp-33, Thr-34, His-38, Trp-40, and Gln-41 and side chain resonances of Trp-33, Asn-35, Trp-40, and Gln-41), the residues in the β5/β6 loop to the β6-β7 sheets (His-79, Cys-84, Arg-85, Phe-86, Asp-87, Ile-88, Ser-93, Trp-95, Tyr-96, and Arg-98), and part of the C-terminal helix (Gln-107, Glu-113, and Gln-114) were strongly affected (Figs. 3B and 6). The mapping of these residues indicates that the membrane interaction surface of the CERT PH domain extends through the entire region of the basic groove, including the PtdIns(4)P-binding site located at the exposed end as well as the basic “side face” of the CERT PH domain (Fig. 3D).

The TCS experiments were also performed with PtdIns(4)P-free liposomes (supplemental Fig. S3). As expected from the lower binding affinity to the PtdIns(4)P-free liposomes (Kₐ ~ 220 μM), the absolute values of the signal intensity reductions were smaller than those obtained from the experiments with the PtdIns(4)P-embedded liposomes. In this experiment, the side face of the CERT PH domain was preferentially saturated (supplemental Fig. S3). This suggests that the side face forms a nonspecific membrane interaction surface, besides the specific PtdIns(4)P-binding site at the exposed end of the CERT PH domain.

Alanine-scanning Mutageneses—To confirm the importance of the basic groove in the interaction with the Golgi membrane, we performed a series of alanine-scanning mutageneses. Single alanine substitutions were introduced for 18 amino acids in the basic groove of the CERT PH domain (Fig. 4 and supplemental Table S1), indicating that those mutations are localized on the edge of the Golgi-binding interface and thus do not show significant energetic contributions to the binding.

Intracellular Localization of CERT Mutants—To confirm our new finding in the CERT PH domain-Golgi membrane interaction, we performed immunofluorescence microscopy experiments using living HeLa cells and verified intracellular localization of the CERT mutants, K32A, W33A, and R43A, which consist of the PtdIns(4)P-specific site and the nonspecific basic binding site at the exposed end of the CERT PH domain (Fig. 3D).

Statistics for the 20 final NMR structures of the CERT PH domain

| Distance restraints                      | NMR distance and dihedral restraints |
|-----------------------------------------|--------------------------------------|
| NOE upper distance restraints           | 1205                                 |
| Short range (|i| - |j| = 1) |                                      |
| Medium range (|i| - |j| < 5)  | 178                                  |
| Long range (|i| - |j| > 5)  | 521                                  |
| Hydrogen bonds                          | 52                                   |
| Total dihedral angle restraints         |                                       |
| ϕ                                       | 58                                   |
| ψ                                       | 56                                   |

* Ramachandran analysis was performed for all residues, using the program PROCHECK NMR.
* The disallowed residue is Gly-64, which is located in the unstructured loop connecting β3 and β4.
* Pairwise r.m.s.d. was calculated for the ordered secondary structure components (amino acid residues 26–29, 43–47, 52–55, 66–70, 75–77, 85–90, 94–98, and 106–115) of the CERT PH domain.

FIGURE 1. Solution structure of the CERT PH domain, determined by NMR. A, primary structure of CERT; B, backbone superposition of the 20 lowest energy structures; and C, ribbon diagram of the representative structure with the lowest energy. These molecular diagrams were generated with the program MOLMOL (26). D, electrostatic potential surface of the CERT PH domain. Red and blue indicate negative and positive electrostatic surfaces, respectively. The electrostatic potential was calculated using the program MOLMOL (26). The “front view” shows the same side as in B and C. E, ribbon model representation of the CERT PH domain, with magnification of the basic groove. The side chains of the basic and aromatic residues in the basic groove are displayed as blue and green sticks, respectively.
Structural Dynamics in the β1/β2 Loops—The FAPP1 PH domain also recognizes PtdIns(4)P (36). A comparison of the tertiary structures between the CERT PH domain and the FAPP1 PH domain suggested that the dynamic nature of the β1/β2 loop region could be different (supplemental Fig. S5A). In the case of the FAPP1 PH domain, the signals from the β1/β2 loops in the FAPP1 PH domain were significantly perturbed by the titration of diC4-PtdIns(4)P, suggesting that the dynamic nature of this loop region in the FAPP1 PH domain was different from that in the CERT PH domain.
Structure and Interaction of CERT PH Domain

A

R.F. irradiation

PtdIns(4)P

Liposome

12C, 1H

15N, 1H

12C, 2H

(bound state)

/free state

CERT PH domain

B

Irradiation (-)  Irradiation (+)

Asp92  Asp92

Gln41  Gln41

15 N (ppm)

1H (ppm)

8.6  8.4

8.6  8.4

C

Intensity reduction ratio

( Irradiation(-) : Irradiation(+))

0.8

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

Residue Number

30  40  50  60  70  80  90  100  110

Side-chain

D

Front-view  Right-view  Back-view
loop region were observed, and the loop was structurally well defined. In contrast, the backbone NMR signals from Asn-35, Tyr-36, Ile-37, and Gly-39 of the CERT PH domain were broadened beyond the detection limit. The amide resonances of Thr-34 and His-38, as well as the side chain NH$_2$ signals of Asn-35, were observed but severely broadened, suggesting that the $\beta_1/\beta_2$ loop undergoes a chemical exchange process. The severely broadened NMR signals of the $\beta_1/\beta_2$ loop region remain invisible with saturated amount of diC$_{4}$-PtdIns(4)P.

Although the amino acid sequences of the $\beta_1/\beta_2$ loop itself is highly conserved between these two PH domains, a pair of residues in the neck of the $\beta_1/\beta_2$ loop, corresponding to Ser-31 and Asp-42 in the CERT PH domain, are different. In the FAPP1 PH domain, these residues are substituted with tyrosine and proline, respectively (supplemental Fig. S5A). Because the $\beta_1/\beta_2$ loop of the CERT PH domain does not interact with the other parts of the proteins, we assumed that the two amino acids might be responsible for the difference in the dynamic nature of the $\beta_1/\beta_2$ loops.

To test this notion, structural dynamics of the $\beta_1/\beta_2$ loop of the CERT PH domain, we introduced the S31Y/D42P mutations to the CERT PH domain. The mutations mimic the structural character of the FAPP PH domain. As a result, the backbone amide signals of Ile-37 and Gly-39, which are severely broadened, became detectable in the two-dimensional $^1$H-15N HMQC spectrum of the S31Y/D42P mutant (supplemental Fig. S5B). Furthermore, $^{15}$N R$_2$ relaxation dispersion experiments revealed that the structural fluctuation on the microsecond to millisecond time scale in the $\beta_1/\beta_2$ loop region of the CERT PH domain was largely suppressed by the S31Y/D42P mutation (supplemental Fig. S5C). These data suggest that the structural dynamics of the $\beta_1/\beta_2$ loop is at least partially regulated by the two amino acid residues located in the neck of the loop.

DISCUSSION

The PH domain in the CERT protein plays a central role in the ER-to-Golgi transport of ceramide, which bound to the parental protein (4). To elucidate the structural basis of the PH domain-dependent Golgi association of the CERT protein, we utilized SPR along with solution state NMR analyses.

The SPR analyses, using the phosphoinositide-embedded liposomes, confirmed that the CERT PH domain specifically recognizes the PtdIns(4)P-embedded Golgi-mimetic liposome with a few micromolar affinity, while showing considerably lower affinities for the liposomes, containing either PtdIns(3)P, PtdIns(5)P or PtdIns(4,5)P$_2$ (Table 1). Considering the localization of PtdIns(4)P in the Golgi membrane, a specific PtdIns(4)P recognition by the CERT PH domain would be reasonable to allow the molecule to associate with the Golgi membrane. The dissociation rate of the CERT PH domain from the PtdIns(4)P-embedded liposomes was rather fast, on the order of s$^{-1}$, as indicated by the box-shaped SPR sensorgram (supplemental Fig. S5D). Thus, the average dwell time of the CERT protein on the Golgi membrane is intrinsically short unless the interaction of the other domain or elements in the CERT protein, such as the START domain, enhances the binding to the Golgi membrane.

Interestingly, the titration of the water-soluble analog of PtdIns(4)P, diC$_{4}$-PtdIns(4)P, revealed that the affinity between the CERT PH domain and the headgroup of PtdIns(4)P is quite weak (Fig. 2B). This is markedly different from the typical phosphoinositide recognition by other PH domains, in which most of the binding energy is derived from the specific interaction with the headgroup (31). In supplemental Fig. S6, tertiary structures of the CERT PH domain were compared side-by-side as well as by superimposition with other PH domains from Grp1 and DAPP1. The CERT PH domain only have two basic residues, Lys-32 and Arg-43, in the PtdInsP binding pocket, which is significantly less compared with Grp1 and DAPP1 PH domains. This might be one of the reasons for the weak headgroup recognition by the CERT PH domain. Assuming that the structure of the CERT PH domain would not change significantly upon the binding to PtdIns(4)P and that the position of PtdIns(4)P in the complex is structurally similar to the other PH domains, both basic residues in the CERT PH domain seem to be directed to the phosphate group of PtdIns(4)P. The residue corresponding to Arg-43 recognizes phosphate in the third position (P3) in other PH domains; however, the difference in the relative position between Arg-43 and Tyr-54 force Arg-43...
toward P4. This also seems to make the binding to P3 hindered by the steric clash between the side chain of Arg-43 and P3 in the CERT PH domain. As for the other residues that recognize P1 and P5 in the other PH domains, there is no corresponding residue in the CERT PH domain. These may explain the PtdIns(4)P specificity of the CERT PH domain. The difference in the affinities between the PtdIns(4)P-embedded liposome and diC4-PtdIns(4)P strongly indicates that additional interactions are required for the full binding affinity of the CERT PH domain.

To understand the details of this characteristic interaction between the CERT PH domain and the Golgi-mimetic membrane, we determined the tertiary structure of the CERT PH domain by solution NMR techniques (Fig. 1, B and C). The structural determination revealed that the main chain trace of the CERT PH domain resembles a typical PH domain fold (Fig. 1, B and C) and that the basic residues are clustered in the middle of the molecule in the CERT PH domain, forming a characteristic basic groove that encircles the protruding part of the β1/β2 region (Fig. 1, D and E).

The NMR binding experiments suggested that the entire basic groove is responsible for the interaction with the PtdIns(4)P-embedded liposomes (Fig. 3). Although the specific PtdIns(4)P-binding site was successfully mapped to the exposed end (Fig. 2), the side face of the basic groove mainly contributes to the nonspecific phospholipid membrane interaction (supplemental Fig. S3). This indicates that the two regions in the basic groove play rather distinct roles. Interestingly, the specific PtdIns(4)P binding and nonspecific phospholipid membrane interactions contribute almost equally to the binding of the CERT PH domain to PtdIns(4)P-embedded liposomes. The dissociation constant of the CERT PH domain to the soluble form of the PtdIns(4)P determined in this study was 760 μM, which is weaker than the binding to the liposome without PtdIns(4)P (220 μM). The functional role of the nonspecific electrostatic interaction has also been proposed for other PH domains (31). However, the contributions of those nonspecific interactions are less prominent, as compared with that of the CERT PH domain (32, 33). For example, in the case of the GRP1 PH domain, the Kd to PtdIns(3,4,5)P3-embedded liposomes is 50 ± 10 nM, and the affinity was reduced by only 12-fold when the acidic lipid components were eliminated from the liposome (34).

The basic groove is composed of seven basic residues and one aromatic residue (Tyr-96) (Fig. 1E). In addition, there is the hydrophobic/aromatic β1/β2 loop protruding from the middle of the basic groove. It is well known that basic residues contribute to the interaction with the anionic surface of phospholipid membranes by electrostatic attraction (31), and that aromatic residues, such as Trp and Tyr, are usually located at the lipid-water interface in membrane proteins or membrane-associating proteins (35). Thus, the basic groove of the CERT PH domain is suitable to interact with the anionic surface of the Golgi membrane. The interaction between the basic groove and the membrane would orient the β1/β2 loop toward the membrane interior (Fig. 5). The interaction of the basic groove does not seem to be selective for particular anionic phospholipids in the Golgi membrane. The CERT PH domain binds to both Golgi-mimetic PtdIns(4)P-free liposomes, which containing 5% of PS, and the PA-containing liposomes, which replace PS in the PtdIns(4)P-free liposomes with PA. Response induced by 16 μM of the CERT PH domain in SPR analysis was 230 and 311 resonance units for phosphatidylserine and phosphatidic acid-containing liposomes, respectively. Stronger binding to PA-embedded liposome was somewhat unexpected; however, the exposed phosphate groups in PA can be the reason for the higher affinity.

Based on these observations, we proposed a model that describes the PH domain-mediated Golgi association of the CERT protein. The initial membrane association of the CERT PH domain might be driven by a nonspecific electrostatic interaction between the basic groove and the phospholipid membrane (Fig. 5B). The interaction using the basic groove with the aromatic residues would orient the β1/β2 loop of the CERT PH domain toward the membrane, and the binding mode would be suitable for the efficient recognition of PtdIns(4)P in the membrane (Fig. 5C). The site-specific mutagenesis analyses further support this binding mode, as they revealed that most of the basic and hydrophobic residues in the basic groove and the β1/β2 loop play critical roles in binding to the PtdIns(4)P-embedded membrane (Fig. 4). The proposed CERT PH domain-Golgi membrane binding mode was confirmed by immunofluorescence microscopy experiments using HeLa cells, which verified Golgi mislocalization of the CERT with mutation in the PtdIns(4)P-binding pocket or the nonspecific basic groove (supplemental Fig. S4). It has been shown that the PH domain is the essential determinant for the Golgi localization of CERT, and its START domain has little contribution to Golgi association (4). The result of the immunofluorescence microscopy using living HeLa cells (supplemental Fig. S4) in this study supports this notion by directly showing that both PtdIns(4)P-specific sites and the nonspecific basic groove are responsible for the Golgi localization of CERT.

The FAPP1 PH domain also recognizes PtdIns(4)P (36). The β1/β2 loop of the PH domain reportedly penetrates into the
The binding mode of the FAPP1 PH domain is consistent with our binding model, as the β1/β2 loop of the CERT PH domain should be buried in the membrane (Fig. 5). However, a comparison of the tertiary structures between the CERT and the FAPP1 PH domain as well as the NMR relaxation properties of the CERT PH domain suggested that there is enhanced dynamics in the β1/β2 loop region of the CERT PH domain (supplemental Fig. S5). Sequence comparison and NMR dynamics analysis using a chimera mutant of the CERT PH domain suggested that the difference in the pair of residues in the neck of the β1/β2 loop, corresponding to Ser-31 and Asp-42 in the CERT PH domain, would at least partially regulate the dynamics of the difference in the dynamic nature of the β1/β2 loops (supplemental Fig. S5). In the FAPP1 PH domain, these residues are substituted with tyrosine and proline, respectively (supplemental Fig. S5A), whereas other residues in the β1/β2 loop is highly conserved between these two PH domains.

The difference in the dynamics might also be reflected in the biological functions of the CERT and FAPP1 PH domains. The FAPP1 and FAPP2 PH domains initiate membrane tubule formation in a PtdIns(4)P-dependent manner and are required for Golgi-to-plasma membrane vesicular transport (36–39). The rigid β1/β2 loop of the FAPP1 PH domain is considered to “wedge” into the Golgi and to reshape the lipid bilayer into a tubule form by increased membrane surface pressure (36). However, the CERT PH domain binding reportedly has no...
effect on the membrane structure. The structurally disordered β1/β2 loop in the CERT PH domain is clearly not appropriate to wedge into the membrane, but it might “lasso” the lipids without affecting the membrane structure. Therefore, it would be interesting to investigate the dynamics of the β1/β2 loops of PH domains, to see if the dynamics of the region coincide with certain functions of the PH domains.

As we mentioned above, the basic groove, composed of Lys-32, Arg-43, Lys-56, Arg-66, His-79, Arg-85, and Arg-98, seems to be primarily important for the recognition of PtdIns(4)P in the Golgi membrane. Among the basic residues in the basic groove, the residues corresponding to Lys-32 and Arg-43 in the CERT PH domain are highly conserved within most of the PH domains, as indicated in the multiple sequence alignment shown in Fig. 6. These residues specifically recognize the phosphate group of phosphatidylinositol (40, 41), and thus the conservation throughout the PH domains would be reasonable. Moreover, the rest of the basic amino acids except for Lys-56, which was proved to be nonessential in the CERT PH domain, are conserved only among the specific types of PH domains that were classified in the top four rows of the sequence alignments. The four PH domains are referred to as the COFs (acronym for CERT, OSBP, and FAPPs) family (Fig. 6) (42, 43). All of the COFs family proteins specifically recognize PtdIns(4)P and target the Golgi in a PtdIns(4)P-dependent manner (4, 27, 39, 40, 44 – 46). In addition, the binding mode of all of the COFs family PH domains are similar to that of the CERT PH domain, as characterized the weak affinities to the headgroup of PtdIns(4)P (the \( K_D \) values are millimolar range or even undetected) and significant affinities to the PtdIns(4)P-embedded Golgi-mimetic liposomes (the \( K_D \) values are 0.3–3 \( \mu \)M) (27, 36, 45). Furthermore, all of the COFs family proteins are known to mainly function in the lipid transport to the organelle, and thus the characteristic binding mode might be suitable to perform this role. The PH domains that recognize bi- or tri-phosphate forms of phosphoinositides (41) lack the basic residues that we proposed to be conserved within the COFs family proteins (Fig. 6). Interestingly, these PH domains have distinct functions, as compared with those of the COFs family PH domains, and contribute to specific signal transduction. This indicates that the interaction modes shared within the subsets of PH domains, including the CERT PH domain, are closely related to their functional characteristics.

In summary, we determined the solution structure of the CERT PH domain, and we identified the PtdIns(4)P-binding site and the membrane-binding interface by solution NMR techniques. The results were supported by SPR-binding experiments, combined with site-specific mutagenesis analyses \textit{in vitro} and in living cells. Based on the observations, we proposed a model in which the rather nonspecific electrostatic interaction between the basic groove and the phospholipid membrane would additionally contribute to the specific interaction with PtdIns(4)P.

The primary sequence alignment indicated that most of the residues located on the basic groove identified in this study are highly conserved within the COFs family but not in other PH domains. Therefore, the nonspecific membrane binding by the basic groove might be a unique characteristic of the COFs family PH domains.

In addition, most of the COFs family proteins are lipid transport proteins that are associated with the Golgi in a PtdIns(4)P-dependent manner (36, 37, 47). However, the proteins involved in signaling are usually specific to the di- or triphosphate forms of phosphoinositides (48 – 51). The signaling proteins require higher specificity and stability. For this purpose, relying on the specific interaction with a multiphosphorylated inositide might be preferred. In contrast, the low affinity recognition of the mono-phosphoinositide headgroup might be favored by lipid transport proteins, because the rather nonspecific scanning of the intracellular membrane is more important for these molecules. The initial membrane binding phase might effectively reduce the effective volume of the space, allowing the lipid transport proteins to find PtdIns(4)P more efficiently on the membrane surface.

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