Phosphoinositides (PIs) are crucial lipid components of membranes and are involved in a number of cellular processes through interactions with their effector proteins. Recently, we have established a lipid-protein nanoscale bilayer (nanodisc) containing PIs, hereafter referred to as PI-nanodisc and demonstrated that it could be used for both qualitative and quantitative evaluations of protein-membrane interactions. Here, we report further NMR analyses for obtaining structural insights at the residue-specific level between PI-binding effector protein and PI-nanodisc, using the FYVE domain of early endosome antigen 1 (EEA1), denoted as EEA1 FYVE, and PI(3)P-nanodisc as a model system. We performed a combination of the NMR analyses including chemical shift perturbation, transferred cross-saturation, and paramagnetic relaxation enhancement experiments. These enabled an identification of the interaction surface, structural change, and relative orientation of EEA1 FYVE to the PI(3)P-incorporated lipid bilayer, substantiating that NMR analyses of protein-membrane interactions using nanodisc makes it possible to show the residue-specific interactions in the lipid bilayer environment.

Phosphoinositides (PIs) are membrane components that play crucial roles in different physiological processes through

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**Background:** Interaction between phosphoinositides (Pis) and effector proteins has not been fully elucidated in a lipid bilayer environment.

**Results:** The interaction of EEA1 FYVE with a PI(3)P-incorporated lipid bilayer was studied by solution NMR techniques.

**Conclusion:** EEA1 FYVE bound to PI(3)P was determined in a physiological condition.

**Significance:** Water-soluble membrane mimetics and solution NMR techniques are powerful tools for elucidating the protein-membrane interactions.

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Phosphoinositides (Pis) are crucial lipid components of membranes and are involved in a number of cellular processes through interactions with their effector proteins. Recently, we have established a lipid-protein nanoscale bilayer (nanodisc) containing PIs, hereafter referred to as PI-nanodisc and demonstrated that it could be used for both qualitative and quantitative evaluations of protein-membrane interactions. Here, we report further NMR analyses for obtaining structural insights at the residue-specific level between PI-binding effector protein and PI-nanodisc, using the FYVE domain of early endosome antigen 1 (EEA1), denoted as EEA1 FYVE, and PI(3)P-nanodisc as a model system. We performed a combination of the NMR analyses including chemical shift perturbation, transferred cross-saturation, and paramagnetic relaxation enhancement experiments. These enabled an identification of the interaction surface, structural change, and relative orientation of EEA1 FYVE to the PI(3)P-incorporated lipid bilayer, substantiating that NMR analyses of protein-membrane interactions using nanodisc makes it possible to show the residue-specific interactions in the lipid bilayer environment.
Nanodisc as a model for study of membrane-protein interaction using solution NMR techniques.

Structural analyses using soluble PI(3)P (17) and the NMR-based approach using a micelle system (6) proposed the models of EEA1 FYVE bound to a PI(3)P-embedded membrane, however the relative orientations of the EEA1 FYVE to the membrane were not consistent in these two studies. Moreover, to the best of our knowledge, no experimental data have been reported to validate the binding mode of EEA1 FYVE with PI(3)P in the lipid bilayer system. In the present study, PI(3)P-nanodisc was applied to characterize the physiological features of the interaction between EEA1 FYVE and PI(3)P embedded in a lipid bilayer environment, which led to a model consistent with the relative orientation suggested by the crystal structure of the dimeric C-terminal domain of EEA1 containing the coiled-coil and FYVE domains (17). Thus, the evaluation of the membrane-protein interactions shown by nanodisc-based NMR analyses is a feasible approach for validation of physiological PI-effector protein interactions.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The MSP was prepared as previously described (16). Briefly, the fragment encoding MSP derived from human apoA-I (residues 56–243) was cloned into pGBHPS (18) and expressed at 28 °C using the Escherichia coli BL21(DE3) strain as a fusion protein with GB1 and hexahistidine tag at the N terminus followed by the HRV-3C protease of the GB1 and hexahistidine tag by HRV-3C protease, followed by the cleavage of the GB1 and hexahistidine tag by HRV-3C protease, and then gel filtration chromatography on a HiLoad 16/60 Superdex 75 pg column (GE Healthcare).

The EEA1 FYVE domain from humans (residues 1347–1411) was expressed in the E. coli BL21(DE3) strain at 25 °C as a fusion protein with GST. The uniformly 2H,15N-labeled EEA1 FYVE for TCS experiments was prepared by growing E. coli host in M9 minimal medium containing [U-2H]glucose and [15N]ammonium chloride in 99% 2H2O. The MSP was purified by Ni2+ affinity chromatography using COSMIOGEL His-Accept (Nacalai tesque), followed by the cleavage of the GB1 and hexahistidine tag by HRV-3C protease, and then gel filtration chromatography on a HiLoad 16/60 Superdex 75 pg column (GE Healthcare).

**Preparation of Nanodisc**—The 1,2-Dimyristoyl-sn-glycerol-3-phosphocholine (DMPC), 1-(1,2-dihexadecanoylphosphatidyl)inositol-3-phosphate (PI(3)P), and 1,2-dipalmitoyl-sn-glycerol-3-phopho(tempo)choline (TEMPO-PC) were purchased from Sigma, Cayman Chemicals, and Avanti Polar Lipids, respectively. The nanodisc was prepared as described elsewhere (16). Briefly, detergent-solubilized lipid micelle and MSPs were incubated, followed by the removal of detergent by Bio-Beads SM-2 (Bio-Rad), and then separated as a single peak by gel filtration chromatography on a Superdex 200 GL 10/300 column (GE Healthcare).

**Lipid compositions used in this study were** DMPC, DMPC mixed with PI(3)P at a molar ratio of 96:4, and DMPC mixed with TEMPO-PC and PI(3)P at a molar ratio of 86:10:4 for the PI-free nanodisc, PI(3)P-nanodisc, and TEMPO-PI(3)P-nanodisc, respectively. The optimal molar ratio of lipid and MSP for the preparation of nanodisc was determined to be 55:1. Uniformly 2H-labeled MSP was utilized to prepare the nanodisc used for the TCS experiments.

**NMR Analyses**—All the NMR experiments were carried out at 298 K on a Varian Inova 600-MHz spectrometer equipped with a cryogenic probe or a 500-MHz spectrometer. The NMR data were processed using the NMRPipe program package (19) and the Sparky program (Sparky 3, University of California, San Francisco).

The error bars for the reduction and the intensity ratios of the signals in the chemical shift perturbation (CSP) experiments of the nanodisc, TCS, and paramagnetic relaxation enhancement (PRE) experiments were calculated based on the signal-to-noise ratios calculated by the Sparky program. The normalized CSP upon addition of inositol 1,3-bisphosphates (Ins(1,3)P2) (Echelon) was calculated by \((\Delta \delta_{1H})^2 + (\Delta \delta_{15N}/6.5)^2\) (20). The error bars of the CSP values were calculated based on the digital resolution by the formula: \(2 \times (\Delta \delta_{1H} \times R_{1H} + \Delta \delta_{15N} \times R_{15N}/6.5^2)\)/CSP, where \(R_{1H}\) and \(R_{15N}\) are the digital resolutions in ppm in the 1H and 15N dimensions, respectively. The dissociation constant \((K_d)\) was estimated by a nonlinear least squares analysis using GraphPad Prism software (GraphPad Prism Software, Inc.) and the equation,

\[
\text{CSP}_{\text{obs}} = \text{CSP}_{\text{sat}} \times \frac{([\text{Ins}(1,3)P_2]_{\text{tot}} + [\text{EEA1 FYVE}]_{\text{tot}} + K_d}{([\text{Ins}(1,3)P_2]_{\text{tot}} + [\text{EEA1 FYVE}]_{\text{tot}} + K_d)^2 - 4 \times ([\text{Ins}(1,3)P_2]_{\text{tot}} + [\text{EEA1 FYVE}]_{\text{tot}})^2}/(2 \times [\text{EEA1 FYVE}]_{\text{tot}})
\]

where \(\text{CSP}_{\text{obs}}\) is the observed CSP at the given total \(\text{Ins}(1,3)P_2\) concentration, \(\text{CSP}_{\text{sat}}\) is the CSP at saturation, and \([\text{EEA1 FYVE}]_{\text{tot}}\) and \([\text{Ins}(1,3)P_2]_{\text{tot}}\) are the total concentrations of EEA1 FYVE and \(\text{Ins}(1,3)P_2\), respectively.

In the CSP experiments, the uniformly 15N-labeled EEA1 FYVE at the concentration of 100 and 180 \(\mu\)M was mixed with various amounts of \(\text{Ins}(1,3)P_2\), and nanodisc, respectively. The buffer solution consisted of 20 mM Tris-HCl (pH 6.8), 100 mM NaCl, 2 mM DTT, and 0.1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in 90% 1H2O, 10% 2H2O. For the interaction with \(\text{Ins}(1,3)P_2\), aliquots of 2.5 mM stock solution of \(\text{Ins}(1,3)P_2\) were sequentially added to the EEA1 FYVE solution at the molar ratios \((\text{EEA1 FYVE}/\text{Ins}(1,3)P_2)\) of 1:0, 1:0.1, 1:0.2, 1:0.5, 1:1, 1:2, 1:3, 1:4, and 1:5, and a \(^{1}H,^{15}N\) HSQC spectrum was recorded at each \(\text{Ins}(1,3)P_2\) concentration. For the interaction with nanodisc, samples containing EEA1 FYVE and nanodisc at molar ratios \((\text{EEA1 FYVE}/\text{nanodisc})\) of 1:0, 1:0.0025, 1:0.005, 1:0.01, and 1:0.02 were individually prepared, and a \(^{1}H,^{15}N\) HSQC spectrum was recorded for each sample.

The TCS experiments were carried out as described elsewhere (21) with minor modifications. The uniformly 2H,15N-labeled EEA1 FYVE at the concentration of 400 \(\mu\)M were prepared in the absence and presence of nanodisc at the molar ratio \((\text{EEA1 FYVE}/\text{nanodisc})\) of 1:0.025. The buffer solution...
consisted of 10 mM Tris-d11 (pH 7.4), 100 mM NaCl, 1 mM d-DTT, and 0.1 mM DSS in 20% 1H2O, 80% 2H2O. The selective saturation for the aliphatic protons of lipid molecules within nanodisc was performed with a WURST-2 pulse of 1500 Hz width centered at 1.5 ppm. The saturation duration and the relaxation delay were set at 1.5 and 5.0 s, respectively.

In the PRE experiments, two samples were prepared under either the paramagnetic or the diamagnetic condition. To prepare the diamagnetic sample, ascorbic acid was added to the sample to a final concentration of 108 µM and incubated at room temperature for 2 h to reduce the TEMPO radical group. The paramagnetic sample was similarly prepared but without ascorbic acid. Both samples consisted of the uniformly 15N-labeled EEA1 FYVE (180 µM) and TEMPO-PI(3)P-nanodisc at the molar ratio (EEA1 FYVE:TEMPO-PI(3)P-nanodisc) of 1:0.005. The buffer solution consisted of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM DSS in 90% 1H2O, 10% 2H2O.

For the assignment of the 1H chemical shifts of DMPC, total correlation spectroscopy (TOCSY) and double quantum filtered-correlation spectroscopy (DQF-COSY) experiments were carried out for 0.2 mM nanodisc composed of DMPC. The buffer solution consisted of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM DSS in 10% 1H2O, 90% 2H2O.

RESULTS

Interaction of PI(3)P-nanodisc and Ins(1,3)P2 with EEA1 FYVE by CSP Experiments—We have previously confirmed the specific binding of EEA1 FYVE to PI(3)P-nanodisc by pulldown binding assay (16). To obtain further insights into the interaction at a residue-specific level, the NMR spectra of EEA1 FYVE upon addition of PI(3)P-nanodisc and Ins(1,3)P2, a head group of PI(3)P, were acquired.

In the presence of up to a 0.02 equivalent molar ratio of PI(3)P-free nanodisc to EEA1 FYVE, the chemical shift changes in the signals of EEA1 FYVE were <0.01 ppm and no residue-specific intensity reduction of the signals was observed, indicating that there were no detectable interactions between the EEA1 FYVE and PI(3)P-free nanodisc (Fig. 1, a and b, left). However, upon addition of PI(3)P-nanodisc, there was a significant intensity reduction for specific residues, indicating the specific binding of EEA1 FYVE to PI(3)P-nanodisc (Fig. 1, a and b, right). Generally, the intensity reduction of the signal upon binding to large molecules is caused by slower tumbling due to increases in the molecular weight of the complex and/or by an intermediate to slow exchange process in the NMR time scale between the free and bound states (22). Because the molecular weight of EEA1 FYVE and PI(3)P-nanodisc are 8000 and ~150,000, respectively, the complex is too large to observe a signal in the 1H-15N HSQC spectrum. This suggests that the uniform intensity reduction of all the EEA1 FYVE signals in the presence of PI(3)P-nanodisc reflects the increase in molecular weight and that the further intensity reduction would be caused by the exchange broadening effect in the residues due to chemical shift changes upon binding. Because the signal derived from Trp1349, which was most significantly affected, disappeared upon addition of 0.01 equivalent molar ratio of PI(3)P-nanodisc to EEA1 FYVE, we estimated the intensity reduction of the signals at 0.005 equivalent molar ratio (Fig. 1c). Considering that the lipid used in the preparation of nanodisc contained DMPC and PI(3)P at a molar ratio of 96:4 and single nanodisc contains 154 lipid molecules (15), on average, three molecules of PI(3)P are included within each leaflet of PI(3)P-nanodisc. Therefore, the 0.005 equivalent molar ratio of PI(3)P-nanodisc to EEA1 FYVE would be a 0.03 equivalent molar ratio of PI(3)P to EEA1 FYVE.

The residues with intensity reduction ratios >0.40 are Trp1349–Asn1353, Val1355, Val1367, Thr1368, and His1372; those in the 0.30–0.40 range are Gln1354, Trp1356, Phe1365, Ser1366, Ala1383, and Arg1406; and those in the 0.25–0.30 range are Met1390, Val1369–Arg1371, His1372, and Arg1375 (Fig. 1c). These residues form a specific surface on the EEA1 FYVE, whereas the residues on the opposite surface are not affected (Fig. 1d). This indicates that the affected residues are involved in direct binding to PI(3)P embedded in membrane and/or are an indirect effect due to conformational changes in EEA1 FYVE.

The CSPs upon binding to Ins(1,3)P2 indicates the residues involved in the recognition of the inositol head group of PI(3)P. Several residues exhibited chemical shift changes in the concentration dependent manner of Ins(1,3)P2 (Fig. 2a). The residues with CSPs >0.5 ppm are Trp1349/Glu1351, Asn1353, Val1355, and His1372, those in the range of 0.2–0.5 ppm are Ala1350, Asp1352, Glu1354, Gln1356, Met1359, Ser1366, Arg1370, Arg1372, His1373, Cys1374, Asn1379, and Phe1381, and those in the range of 0.1–0.2 ppm are Phe1365, Thr1368, Arg1375, Gly1376, Ile1380, Ala1383, Ala1387, Ala1390, Arg1400, and Cys1402 (Fig. 2b). These residues formed a specific surface involving the binding site of the inositol head group of PI(3)P shown from the crystal structure (Fig. 2c). The observed CSPs were mostly consistent with those previously reported using EEA1 FYVE containing an additional 21 residues at the N terminus and a water-soluble PI(3)P analog with short acyl chains: diC4-PI(3)P, indicating that the recognition of the inositol head group of PI(3)P is independent of the presence of the additional preceding residues in EEA1 FYVE and short acyl chains of diC4-PI(3)P (6). The Kd value for Ins(1,3)P2 binding to EEA1 FYVE was estimated to be ~105 µM. The result of CSP experiment using PI(3)P-nanodisc (Fig. 1c) is summarized below the residue number in Fig. 2b, for comparison. The residue that was not significantly affected upon binding to Ins(1,3)P2 (Fig. 2a) but was significantly affected upon binding to PI(3)P-nanodisc (Fig. 1a) was Val1367, indicating that at least Val1367 was involved in the interaction with the membrane (Fig. 2, b and c).

Determination of the Residues on EEA1 FYVE Proximal to PI(3)P-nanodisc in the Complex by TCS Experiments—To determine the residues on EEA1 FYVE directly interacting with PI(3)P and/or the surrounding lipids, we performed TCS experiments (Fig. 3a). The TCS is an NMR method to determine the direct interaction surface of large protein-protein complexes (21, 23), and the TCS method has also been successfully applied to the membrane-protein interactions using liposomes (9, 24, 25) and bicelles (26). Preliminary to the TCS experiment, the saturation effects for lipid protons of nanodisc were checked. Irradiation at a frequency centered at the aliphatic proton resonances of the tail group of the lipid selectively attenuated the NMR signals of the hydrophobic tail groups by direct saturation. Upon irradiation of the hydrophobic tail, the NMR signals of the polar head.
group, choline methyl signal at the frequency of 3.2 ppm, was not effectively attenuated so that saturation was not effectively transferred to the head group, probably due to the high mobility of choline head group. Low efficient saturation of choline methyl and glycerol protons of phospholipid has similarly been reported for bicelles (26). To maximize the saturation efficiency of the protons in nanodisc without affecting the water signal, we used the WURST-2 pulse of 2.5 ppm width centered at 1.5 ppm in this study. This resulted in a complete reduction of aliphatic signals in the hydrophobic tail and a 25–30% reduction of the signal intensity in the choline methyl protons of DMPC, respectively (Fig. 3, b and c).

Next, TCS measurements based on the above irradiation experiments were made. Significant intensity reductions were observed for several amide signals of EEA1 FYVE in the presence of PI(3)P-nanodisc by irradiation at the aliphatic group of the lipid (Fig. 3d). Two different control samples in the presence of PI-free nanodisc and in the absence of nanodisc were used.

**FIGURE 1.** Spectral changes in the CSP experiments of EEA1 FYVE and PI(3)P-nanodisc. a, portion of $^1$H-$^1$H HSQC spectra in the presence of 0 (black), 0.005 (blue), 0.01 (yellow), and 0.02 (red) molar equivalents of PI-free nanodisc (left) and PI(3)P-nanodisc (right) overlaid, respectively. b, one-dimensional slices of Val$^{1367}$ and Glu$^{1384}$ signals in the presence of various amounts of PI-free nanodisc (left) and PI(3)P-nanodisc (right) overlaid, respectively. c, plot of difference in reduction ratios ($\Delta$RR) of each signal in the presence of the 0.005 molar equivalents of nanodisc. The $\Delta$RR value of each signal was calculated by the equation: $\Delta$RR = RR$_{\text{PI(3)P-nanodisc}}$ – RR$_{\text{PI-free nanodisc}}$, where RR$_{\text{PI(3)P-nanodisc}}$ and RR$_{\text{PI-free nanodisc}}$ indicate the intensity reduction ratio of the signal in the presence of PI(3)P-nanodisc and PI-free nanodisc, respectively. Asterisks indicate residues with no data. The error bars were calculated based on the signal-to-noise ratios of each signal. The bars with $\Delta$RR values >0.40 and in the 0.30–0.40 range are red and orange, respectively, and labeled. The bars with $\Delta$RR values in the 0.25–0.30 range are yellow. The secondary structure of EEA1 FYVE is shown below the residue number. d, the residues with significant intensity reductions in the signals upon binding to PI(3)P-nanodisc mapped on the surface of the EEA1 FYVE structure (Protein Data Bank code 1JOC), using the same color representations as in c. Residues with no data are colored black.
disc exhibited similar results, indicating that there is no detectable interaction between EEA1 FYVE and PI-free nanodisc (supplemental Fig. 1a). A relatively large intensity reduction ratio (RR), larger than 0.15, was observed for Lys1388, even in the absence of nanodisc. The intensity reduction for the proximal amide groups would be caused, in principle, by the direct saturation of a small amount of residual protons even in the highly deuterated EEA1 FYVE and/or exchangeable protons in the hydroxyl and thiol groups (27), presumably by the hydroxyl group of Ser1386 and/or the thiol group of Cys1385 in this case (supplemental Fig. 1b). We evaluated the cross-saturation from PI(3)P-nanodisc as a \( \Delta RR \) value, by subtracting the RR value in the presence of PI-free nanodisc from those in the presence of PI(3)P-nanodisc (Fig. 3e). The residues with \( \Delta RR \) values larger than 0.10 are Arg\(^{1370}\)–His\(^{1372}\), and those with \( \Delta RR \) values in the 0.06–0.10 range are Trp\(^{1349}\), Asn\(^{1353}\), Phe\(^{1365}\), Phe\(^{1381}\), Glu\(^{1384}\), Ser\(^{1386}\), and Ala\(^{1387}\). These residues were mostly located on a specific side of the EEA1 FYVE molecule (Fig. 3f), whereas they were discrete within the surface. The residues not affected within the surface are probably due to the low surface accessibility of the backbone amide protons and/or the ineffective saturation transfer from the protons at the polar head group. We concluded that the saturation from the protons at the head group of PI(3)P and/or those at the head group of DMPC would be effectively transferred, and a direct interaction surface on EEA1 FYVE with PI(3)P-nanodisc was successfully determined (Fig. 3f). The relatively small intensity reduction of the EEA1 FYVE signals in the TCS experiment would be partially due to inefficient saturation of the polar head group. Selective saturation of the more specific lipid protons could provide a larger cross-saturation effect, leading to an

**FIGURE 2. Chemical shift perturbation of EEA1 FYVE upon binding to Ins(1,3)P\(_2\).**

(a), \( ^{1}H\)-\( ^{15}N \) HSQC spectra of EEA1 FYVE in the presence of 0 (black), 0.5 (blue), 1.0 (cyan), 2.0 (green), 4.0 (yellow), and 5.0 (red) molar equivalents of Ins(1,3)P\(_2\) overlaid. Side chain signals of Asn and Gln residues are connected by solid horizontal lines and labeled within parentheses. Signals with the normalized CSP values >0.5 ppm upon binding to 5.0 molar equivalents of Ins(1,3)P\(_2\) are also connected by solid lines. The same region as described in Fig. 1a is boxed and enlarged, for ease of comparison. (b), normalized CSP values were calculated by the equation \( \left( \frac{\Delta \delta \left(^{1}H\right)}{\Delta \delta \left(^{15}N\right)/6.5} \right)^{1/2} \) (20) and plotted for each residue on EEA1 FYVE. Asterisks indicate residues with no data. The error bars were calculated based on the digital resolution of the spectra. The bars with CSPs larger than 0.50 and in the 0.20–0.50 range are red and orange, respectively, and labeled. The bars with CSPs in the 0.12–0.20 range are yellow. The result of CSP experiment for PI(3)P-nanodisc evaluated in Fig. 1c is shown below the residue number. The blue dashed box indicated the residue that was not significantly affected upon binding to Ins(1,3)P\(_2\), but was significantly affected by binding to PI(3)P-nanodisc. c, the residues with significant CSPs upon binding to Ins(1,3)P\(_2\) are mapped on the surface of the EEA1 FYVE structure, using the same color representations as in b. The Ins(1,3)P\(_2\) molecule bound to EEA1 FYVE in the crystal structure is shown in sticks. Residues with no data are colored black. The Val\(^{1367}\) residue boxed in b is circled and labeled in parentheses.
unambiguous determination of the membrane interacting surface.

Determination of the Residues Proximal to the Membrane Surface by PRE Effects from the Spin-labeled Lipid Incorporated into PI(3)P-nanodisc—To gain further insights into the relative orientation of EEA1 FYVE to the membrane in the complex with PI(3)P-nanodisc, TEMPO-PC was introduced into PI(3)P-nanodisc as a paramagnetic probe (Fig. 4a). The PREs from TEMPO were evaluated based on the signal intensity ratios of each residue under the paramagnetic condition to those under the diamagnetic condition (Fig. 4b). The residues with intensity ratios $<0.80$ are the side chain imino proton of Trp$^{1349}$ and amide protons of Asn$^{1353}$, Gln$^{1356}$, and Asn$^{1389}$, those in the 0.80–0.90 range are main chain amide protons of Val$^{1367}$ and Thr$^{1368}$ and a side chain amide proton of Gln$^{1356}$, and those in the 0.90–0.95 range are the main chain amide protons of Lys$^{1348}$, Asp$^{1352}$, Gln$^{1356}$, Arg$^{1370}$-Arg$^{1371}$, Glu$^{1384}$, Asn$^{1389}$, Ser$^{1394}$-Ser$^{1395}$, Lys$^{1397}$, and Arg$^{1400}$. Small but significant PREs were observed for several main chain amide protons; however, the larger PREs were observed for some residues in the side chain imino or amide protons, which could be explained by the higher surface accessibility of the side chain.
imino and amide protons than the main chain amide protons. Among nine residues with side chain imino or amide protons, four affected residues were all located on a specific surface, and the other five were located on the opposite surface (Fig. 4d).

The residues with relatively large PREs in the main chain amide protons are also included in the same surface (Fig. 4c), indicating that this surface is close to the membrane in the complex with PI(3)P-nanodisc. The degree of intensity reductions caused by PREs from TEMPO in the main chain amide protons mapped on the surface of the EEA1 FYVE structure, using the color representations in b. Residues with no data are black. d, residues with large intensity reductions in the side chain imino proton of Trp or amide protons of Gln and Asn mapped on the surface of EEA1 FYVE. The residues with significant PREs from TEMPO, observed as intensity ratios smaller than 0.95 in the side chain amide protons, are red and the others are blue.

**DISCUSSION**

**Interpretation of the Spectral Change Observed in the CSP Experiments of PI(3)P-nanodisc**—Spectral change in EEA1 FYVE upon binding to PI(3)P-nanodisc is described here mainly based on the intensity reduction caused by the chemical exchange process between the free and bound states. However, the chemical shift values of the EEA1 FYVE bound to PI(3)P-nanodisc could not be determined due to the large molecular weight of the complex and exchange broadening. The spectral change in EEA1 FYVE upon binding to Ins(1,3)P_2 indicates an intermediate to fast exchange process in the NMR time scale. The affinity between EEA1 FYVE and PI(3)P is dependent on pH, as the protonation level of histidine residues is critical for the PI(3)P recognition. We have previously determined the apparent dissociation constant ($K_{D}^{app}$) for PI(3)P-nanodisc to EEA1 FYVE at pH 6.0 as $\sim 30$ nM by fluorescence polarization measurements (16). Because the affinity is reportedly 10-fold lower at pH 7.4 than at pH 6.0 (28), the $K_{D}^{app}$ values at pH 7.4 and pH 6.8 are estimated to be 300 nM and within the 30–300 nM range, respectively. Thus, the affinity of EEA1 FYVE to PI(3)P-nanodisc is 100–1000-fold higher than that to Ins(1,3)P_2, and thus, the spectral change in EEA1 FYVE upon binding to PI(3)P-nanodisc would be observed as an intermediate to slow exchange process in the NMR time scale.

Considering the composition of the lipid and the amino acid sequence of MSP used for PI(3)P-nanodisc in this study, it may be assumed that three molecules of PI(3)P are involved in each leaflet of the membrane of PI(3)P-nanodisc, on average. The
PI(3)P-nanodisc is a discoidal structure with a diameter of \(~\sim 90\) Å and the maximum length of EE1 FYVE is \(~\sim 40\) Å. In light of the molecular size of EE1 FYVE and PI(3)P-nanodisc, three molecules of EE1 FYVE could simultaneously bind to PI(3)P on each leaflet of the PI(3)P-nanodisc. As shown in Fig. 1c, the intensity reduction ratios of the signals reached more than 0.1 in the presence of 0.005 equivalent molar ratio of PI(3)P-nanodisc to EE1 FYVE, corresponding to a 0.03 equivalent molar ratio of PI(3)P to EE1 FYVE. This intensity reduction ratio is obviously larger than the molar ratio of PI(3)P to EE1 FYVE, indicating that the residues on the interface are broadened due to exchange broadening processes between the bound and free states.

Interpretation of the NMR Data Acquired by the CSP, TCS, and PRE Experiments—As mentioned above in the result session on the CSP experiments, Val\(^{1367}\) was not significantly affected upon binding to Ins(1,3)\(_2\)P\(_2\) (Fig. 2) but was significantly affected upon binding to PI(3)P-nanodisc (Fig. 1), indicating that at least Val\(^{1367}\) was involved in the membrane binding (Fig. 2, b and c). However, not only the residues involved in the direct binding but also those affected by the indirect binding such as a conformational change are detected in the CSP experiment. To distinguish the residues involved in direct or indirect interaction and identify the relative orientation of EE1 FYVE to the membrane, we performed the TCS and PRE experiments. Although these NMR experiments shown in this paper were performed in different pH conditions of pH 7.4 for TCS and pH 6.8 for CSP and PRE experiments, the TCS and CSP experiments at pH 6.8 and pH 7.4, respectively, exhibited similar results, suggesting that the binding residues on EE1 FYVE to PI(3)P-nanodisc were conserved in this pH range (data not shown).

The TCS experiments determined the direct binding surface on EE1 FYVE to PI(3)P incorporated membrane, and the orientation to the membrane was confirmed by the PRE experiments. PREs on Arg\(^{1370}\)–Arg\(^{1371}\) were relatively small, whereas large intensity reductions were observed on these residues in TCS. This would be explained by the position of the paramagnetic center of TEMPO attached to the head group of phosphatidylincholine, which resulted in smaller PREs than those expected from TCS results.

The residues significantly affected upon binding to PI(3)P-nanodisc not in the TCS (Fig. 3e) but in the CSP experiments (Fig. 1c) are Ala\(^{1350}\), Glu\(^{1352}\), Glu\(^{1354}\), Gln\(^{1356}\), Met\(^{1359}\), Ser\(^{1366}\), Val\(^{1369}\), His\(^{1373}\), Arg\(^{1375}\), Ala\(^{1383}\), and Arg\(^{1400}\). Among these residues, Ala\(^{1350}\)–Glu\(^{1352}\), Glu\(^{1354}\)–Gln\(^{1356}\), Met\(^{1359}\), Ser\(^{1366}\)–Val\(^{1369}\), His\(^{1373}\), and Arg\(^{1375}\)–Ala\(^{1383}\) are located in the PI(3)P-nanodisc binding surface, suggesting that they are involved in the conformational change upon binding to PI(3)P-nanodisc. Conformational change upon binding to the head group of PI(3)P has also been observed in the NMR structures of the free (Protein Data Bank code 1HYJ) and Ins(1,3)\(_2\)P\(_2\)-bound (Protein Data Bank code 1HYI) EE1 FYVE (29). The Ser\(^{1366}\), Val\(^{1369}\) are located within the PI(3)P-nanodisc binding surface, and they are assumed to undergo conformational changes as shown in the NMR structures. However, no significant effects were observed for these residues in the TCS experiments, probably due to the ineffective saturation transfer from the protons at the polar head group of the lipid.

The other residues: His\(^{1373}\), Arg\(^{1375}\), Ala\(^{1383}\), and Arg\(^{1400}\) are located in the PI(3)P-binding surface, but no significant effects were observed in the TCS experiments, probably due to the small surface exposure of backbone amide protons and/or the ineffective saturation transfer from the protons at the polar head group of lipid.

Structural Model of the EE1 FYVE Bound to the PI(3)P-embedded Membrane—The EE1 FYVE bound to a PI(3)P-embedded membrane was manually modeled based on the results of TCS analyses, where the backbone amides of Trp\(^{1349}\), Asn\(^{1353}\), Phe\(^{1365}\), Glu\(^{1384}\), Ser\(^{1386}\), and Ala\(^{1387}\), which were located at the rim of the PI(3)P-nanodisc binding surface of EE1 FYVE (Fig. 3f, center), lay roughly parallel to the membrane plane (Fig. 5). Roughly 10-degree rotations relative to the membrane surface would be allowed for consistency with the NMR results. Although Lys\(^{1348}\) at the N terminus of EE1 FYVE, which was affected only in PRE experiment, seems to be a little away from the membrane in the model, it could be explained by the conformational change upon binding to PI(3)P-nanodisc. The N terminus of EE1 FYVE, including Lys\(^{1348}\), is flexible and occasionally become close to the membrane surface in the complex with PI(3)P-nanodisc, which would result in the PRE effect. Therefore, the result of PRE experiment is also consistent with this model.

The previous models based on the crystal structure of dimeric EE1 FYVE and the NMR-based approach using a micelle system are different in the relative orientation to the membrane and the location of the \(\beta/\beta'\) loop of EE1 FYVE. The \(\beta/\beta'\) loop contacts with the head group region of the lipid bilayer in the model based on the crystal structure (17), whereas the \(\beta/\beta'\) loop is distant from the membrane surface in the NMR-based model (6). With respect to the location of the \(\beta/\beta'\) loop in the complex, small but significant PREs were observed for the main chain amide protons of Ser\(^{1394}\), Ser\(^{1395}\), and Lys\(^{1397}\) within the \(\beta/\beta'\) loop in the present study using PI(3)P-nanodisc, which would reflect that these residues are close to the membrane. The resulting model is consistent with that proposed based on the crystal structure of the Ins(1,3)\(_2\)P\(_2\)-
binding dimeric EEA1 FYVE. Alanine mutations in Trp\(^{1349}\) Phe\(^{1365}\), Arg\(^{1370}\), Arg\(^{1371}\), His\(^{1372}\), Arg\(^{1375}\), Gly\(^{1378}\), and Arg\(^{1400}\) of EEA1 FYVE, which are located close to the head group of the PI(3)P, decreased the PI(3)P binding affinity (30), and Val\(^{1367}\)–Thr\(^{1368}\) to GG and EE mutants both lost the PI(3)P-dependent localization to membrane (31), which could be explained by the present model (Fig. 5).

**Application of Nanodisc for Structural Analyses of the Interaction between Protein and Membrane Using NMR**—Several techniques, including electron paramagnetic resonance (32), monolayer penetration technique combined with mutational analyses (33), x-ray reflectivity (34), solid state NMR (35), and solution NMR have been utilized for the elucidation of the interaction between protein and membrane. Notably, solution NMR using TCS and CSP analyses is powerful in distinguishing between the residues involved in direct binding and conformational changes under nearly physiological conditions, without chemical modification of the protein, which may have a significant effect on the interaction with membrane.

Bicelles, liposomes, and nanodisc are commonly used for functional assays as membrane mimetics that possess lipid bilayers. Among these, nanodisc is probably the most suitable as a tool for analyzing membrane-protein interactions in solution as the other systems have potential drawbacks: bicelles include detergents, and liposomes are insoluble in water. The easy quantification based on the absorbance at 280 nm of MSP is a notable advantage for nanodisc in quantitative analyses. Observation of NMR signals of membrane binding proteins complexed with nanodisc is generally difficult due to the relatively large molecular weight of nanodisc. In such a case, NMR techniques such as CSP, TCS, and PRE, which detect macro-molecular complexes in the exchange process between the free and bound states, provide a plausible model of the complex. Finally, it may be concluded that a re-evaluation of the interaction between the protein and membrane under the lipid bilayer condition using nanodisc is a feasible approach for understanding their physiological interaction.

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