Lipid-Anchored Proteins

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The Role of the Prod1 Membrane Anchor in Newt Limb Regeneration

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Abstract: *Prod1 is a protein that regulates limb regeneration in salamanders by determining the direction of limb growth. Prod1 is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor, but the role of membrane anchoring in the limb regeneration process is poorly understood. In this study, we investigated the functional role of the anchoring of Prod1 to the membrane by using its synthetic mimics in combination with solid-state NMR spectroscopy and fluorescent microscopy techniques. Anchoring did not affect the three-dimensional structure of Prod1 but did induce aggregation by aligning the molecules and drastically reducing the molecular motion on the two-dimensional membrane surface. Interestingly, aggregated Prod1 interacted with Prod1 molecules tethered on the surface of opposing membranes, inducing membrane adhesion. Our results strongly suggest that anchoring of the salamander-specific protein Prod1 assists cell adhesion in the limb regeneration process.*

Among tetrapods, salamanders have an exceptional ability to regenerate their limbs. When salamanders lose a limb by amputation, the wound is covered by a layer of epidermis, beneath which blastemal cells are then created. To properly copy the structure of the missing limb, the blastemal cells require positional information.[1] It has been postulated that the salamander-specific protein Prod1 determines the proper proximodistal identity of regenerating limb tissues based on a concentration gradient.[2] Newt Prod1 is composed of five β-strands and one α-helix and is tethered to the blastemal cell membrane by a glycosylphosphatidylinositol (GPI) anchor.[3] Although the anchoring of Prod1 to the membrane is considered to help regulate its local concentration, the functional role of the anchoring remains elusive. Solid-state NMR spectroscopy is a useful tool for elucidating membrane-related biological phenomena,[4] but difficulties in overexpressing lipid-anchored proteins that require complex post-translational modifications have hampered NMR studies. In this study, we therefore prepared a synthetic mimic of lipid-anchored Prod1 in which the protein component was expressed in *E. coli* and attached to a chemically synthesized lipid-anchor mimic.[5] We then examined the protein's physicochemical properties on membranes. Solid-state NMR and microscopic analyses demonstrated that the anchoring of Prod1 has a profound effect on aggregation that may assist the adhesion of blastemal cells.

The soluble protein component of uniformly ^{13}C- and ^{15}N-labeled Prod1 that contained a sortase A recognition sequence and His-tag (LPETGG-His6) at the C-terminus was expressed in *E. coli*. The recombinant protein was then grafted onto a chemically synthesized lipid entity using the sortase A-mediated chemoenzymatic coupling reaction (Figure 1a).[6] We examined the effect of the anchor on the mobility of Prod1 based on 2D ^{1}H–^{15}N refocused insensitive nuclei enhanced by polarization transfer (refocused INEPT) spectra acquired under conditions of 5 kHz magic angle spinning (MAS) at 25 °C (Figure 1b). We expected to observe highly mobile components in the INEPT spectra. Anchorless Prod1 dissolved in NMR buffer (50 mM potassium phosphate, pH 6.0, 200 mM NaCl) (Figure 1b, left) gave a sufficiently high-resolution spectrum, which was comparable to that obtained in solution NMR. However, many signals disappeared after anchoring to bicelles (Figure 1b, right), and only the C-terminal linker region (QLPET in Figure 1a) remained, suggesting that the mobility of Prod1 was restricted. The possibility that the decreasing motion of Prod1 with a lipid anchor is due to the binding to bicelles was ruled out because the 2D ^{1}H–^{15}N INEPT spectrum of anchorless Prod1 mixed with bicelles (Supporting Information, Figure S1) was almost the same as that dissolved in the buffer.

A decrease in the mobility of Prod1 after anchoring was also observed in liposomes. Figure 1c–e shows ^{13}C spectra representing three different ^{13}C solid-state NMR measurements of Prod1 anchored on liposomes acquired using MAS, as follows:[7] refocused INEPT for fast components (Figure 1c),[8] direct polarization (DP) for all ^{13}C atoms regardless of mobility (Figure 1d), and cross polarization (CP) for slow components[9] (Figure 1e). Most of the signals originating

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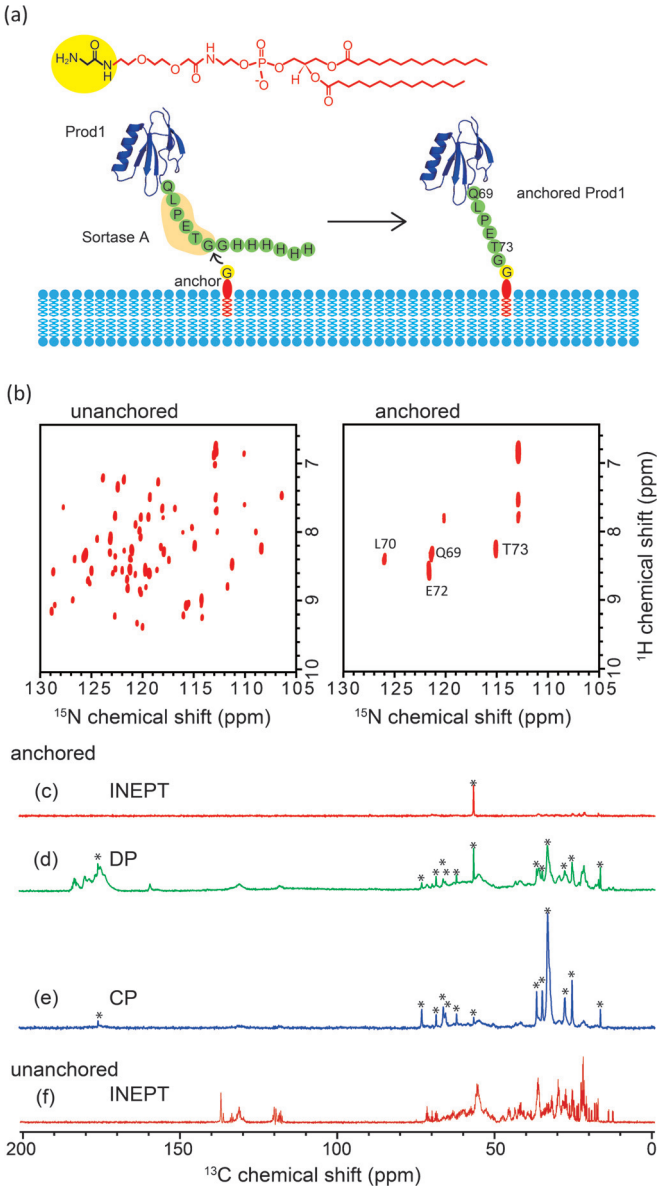

Figure 1. a) Schematic of the chemical structure of the lipid-anchor (3-*O*-(12-glycyl-3,12-diaza-6,9-dioxa-4-oxododecylphosphono)-1,2-*O*-dimyristoyl-*sn*-glycerol) and sortase A-mediated ligation of C-terminal LPETG-tagged Prod1 to the lipid anchor on the membrane. b) 2D ^{1}H–^{15}N refocused INEPT spectra of (left) anchorless Prod1 dissolved in NMR buffer and (right) Prod1 anchored to bicelles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and 1,2- diheptanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC) lipids in 3:1 molar ratio ([Prod1]/[lipid] = 1/300). Spectra in (b) were obtained by recording 100 (left) or 64 (right) t_1 increments with a 231 µs dwell time and 256 (left) or 2048 (right) scans each. c) 1D ^{13}C refocused INEPT, d) DP, and e) CP spectra of Prod1 anchored to DMPC liposomes ([Prod1]/[DMPC] = 1/200). Spectra (c)–(e) were obtained using 30 000 scans and acquisition times of 61 ms (c) and 45 ms (d,e). f) 1D ^{13}C refocused INEPT spectrum of anchorless Prod1 dissolved in NMR buffer. Spectrum (f) was obtained using 45 000 scans and an acquisition time of 61 ms. Peaks from lipids are marked with an asterisk (*). Spectra shown in (b)–(f) were acquired under conditions of 5 kHz MAS at 25 °C.

from Prod1 were negligible in the INEPT spectrum, although they were detected in the CP and DP spectra. In contrast, signals indicative of anchorless Prod1 (Figure 1 f) did not disappear in the INEPT spectrum. We previously reported that anchoring has minimal effect on 1D and 2D INEPT spectra of GB1 (B1 domain of IgG-binding protein G), a standard model protein used in NMR studies (Supporting Information, Figure S2).[5] These results indicate that mobility is restricted only when Prod1 is anchored to the membrane.

To investigate the interactions between Prod1 and lipid membranes, fluorescently labeled Prod1 (Cy7-Prod1) on a micro-patterned supported planar lipid bilayer (SPB) was observed using a total internal reflection fluorescence (TIRF) technique. After a one-hour incubation, diffusion of fluorescent anchored Cy7-Prod1 on the membrane was observed (Figure 2 a), indicating that membrane-anchored Prod1 was mobile. However, large immobile aggregates were observed after 17 h of incubation (Figure 2 b). Although the size of the aggregates depended on the Prod1 concentration and incubation time, the large aggregates were composed of about 250–1700 Prod1 molecules (Figure 2 c). Therefore, we concluded that the disappearance of signals in the INEPT NMR spectra resulted primarily from Prod1 aggregation. The aggregation was specific to membrane-anchored Prod1, as gel filtration chromatography of soluble Prod1 (about 5 mM) showed only one component at the elution time corresponding to the monomer molecular weight of Prod1 (MW = 9.1 kDa; Supporting Information, Figure S3 b,c). In contrast to anchored Cy7-Prod1, anchored Cy7-GB1 did not form aggregates, even after 17 h of incubation (Supporting Information, Figure S4 d–f). The difference in aggregation behavior between Prod1 and GB1 suggests that membrane anchoring is necessary but not sufficient for aggregation.

We then examined the interactions between non-labeled Prod1 anchored on the membrane and anchorless Cy7-Prod1 in solution. Cy7-Prod1 adsorbed onto the membrane only when non-labeled Prod1 was incubated for 17 h before the addition of Cy7-Prod1 (Figure 2 d). This manner of aggregate formation by Cy7-Prod1 is indicative of a trans interaction. Adsorption of Cy7-Prod1 was not observed if non-labeled Prod1 was not pre-added (Figure 2 e) or if Cy7-Prod1 was added immediately after Prod1 anchoring (Figure 2 f). These results suggest that the formation of Prod1 aggregates on the membrane through a cis interaction serves as a platform for the trans interaction between Prod1 molecules on opposing membranes.

We predicted that the trans interaction of anchored Prod1 would result in membrane adhesion. To test this hypothesis, we measured the size of large unilamellar vesicles (LUVs) (diameter: ca. 100 nm) in the presence and absence of anchored Prod1 using dynamic light scattering (DLS, Figure 3). An increase in the size of LUV assemblies was demonstrated by a slower decay of the autocorrelation function in the presence of anchored Prod1 (Figure 3 a) compared with the absence of Prod1. Significant differences in the particle size distribution (Figure 3 b) and mean diameter values (Figure 3 c) were observed. The mean diameter of the control LUVs was 123 nm, whereas two components were detected after anchoring of Prod1 on the LUVs. The first component (x_1; 169 nm) was almost the same size as a single vesicle, but the second component (x_2) was

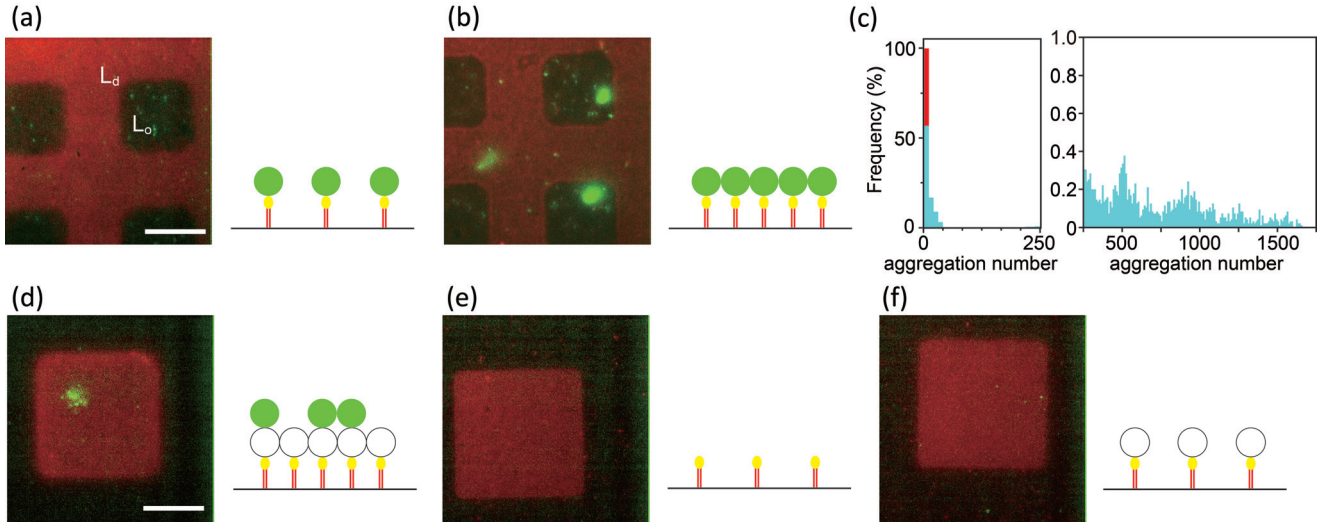

Figure 2. Fluorescence microscopy images of Prod1 on SPBs. a,b) Observation of the *cis* interaction in the presence of anchored Cy7-Prod1. SPBs were composed of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC)/ 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC)/cholesterol/G_{M1} ganglioside (G_{M1})/anchor/rhodamine-1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE). The square and grid-shaped regions were composed primarily of DPPC/cholesterol (L_o phase) and DOPC (L_d phase), respectively. Samples were incubated for (a) 1 h or (b) 17 h after anchoring Cy7-Prod1 onto the membrane. Compared with the original concentration of Cy7-Prod1 in the solution (8.5×10^{-8} M), the number of anchored Cy7-Prod1 molecules on the membrane was kept low to allow single molecule observations by restricting the reaction time with sortase A. c) The histogram shows the aggregation numbers of Prod1 molecules determined from the fluorescence intensities of individual particles. The product of the fluorescence intensity and area was scaled by the average number of particles exhibiting the lowest fluorescence intensity. Incubation time: 1 h (red), 17 h (light blue). d–f) Observation of the trans interaction between non-labeled Prod1 anchored to the membrane and Cy7-Prod1 in solution. The square-shaped fluid bilayer (DOPC/DPPC/cholesterol/G_{M1}/anchor/rhodamine-DOPE) was surrounded by a polymeric bilayer (polymerized 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine bilayer). d) The sample was pre-incubated for 17 h with Prod1 before addition of Cy7-Prod1. e) Prod1 was not pre-added before the 17-h incubation. f) Cy7-Prod1 was introduced immediately after Prod1 anchoring, without incubation. A schematic is added to each micrograph, in which green and white circles represent labeled and non-labeled Prod1, respectively. Scale bars = 10 μm.

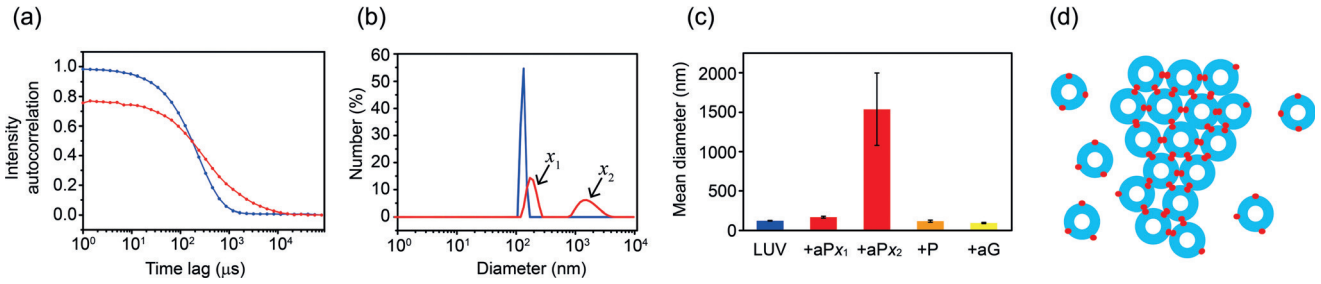

Figure 3. Dynamic light scattering results for DMPC LUVs in the presence (red) and absence (blue) of anchored Prod1([Prod1]/[DMPC] = 1/22) at 30 °C. a) Intensity of autocorrelation and b) particle size distribution plots of results representative of the twenty four measurements. c) Mean hydrodynamic diameter of LUVs corresponding to (from left to right) LUVs, first (+aPx_1) and second (+aPx_2) components of LUVs in the presence of anchored Prod1, LUVs with unanchored Prod1 (+P), and LUVs in the presence of anchored GB1 (+aG). d) Schematic of membrane adhesion mediated by anchored Prod1.

about 10-fold larger (1537 nm) (Figure 3 b,c). The much larger error in the measurement for the second component may reflect the broader size distribution of the LUV clusters. The LUVs did not aggregate even if anchorless Prod1 or anchored GB1 was present. Thus, the results of the DLS measurements provide convincing evidence for the adhesive ability of anchored Prod1.

Next, we examined the conformational conversion of Prod1 during aggregation, as it is thought that GPI-anchoring of prion protein induces a conformational conversion that promotes protein aggregation and the development of neurodegenerative disease.[10] We acquired a 2D ^{13}C–^{13}C dipolar-assisted rotational resonance (DARR) spectrum[11] for Prod1 anchored on liposomes under conditions of 13 kHz MAS at −8 °C with 40 ms of mixing (Figure 4). The chemical shift values in the solution state were sequentially assigned based on solution NMR (Supporting Information, Table S1). From these values, we marked the estimated intra-residue correlations up to three bonds apart using colored circles, as shown in Figure 4. As the experimental values agreed well with the estimated values, we concluded that Prod1 assumes almost the same structure in both the aggregated and in-solution states (Figure 5 a). Therefore, the aggregation mechanism of Prod1 would be expected to differ from that of prion protein.

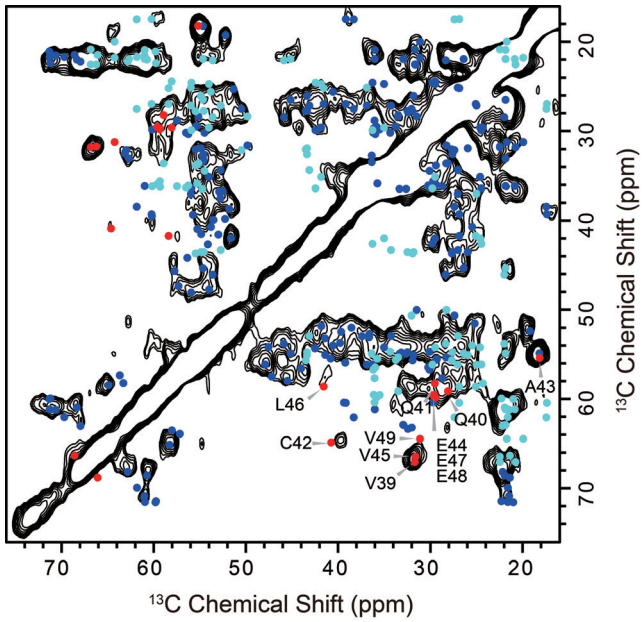

Figure 4. 2D chemical shift correlation spectrum of Prod1 anchored to DMPC liposomes ([Prod1]/[DMPC] = 1/200) with a DARR mixing time of 40 ms, acquired under conditions of 13 kHz MAS at −8 °C. The data size was 265 points with a 13 μs dwell time in t_1 and 2048 complex points with a 13 μs dwell time in t_2. The data was processed with SINE = 3 apodization and 100 Hz exponential line broadening in the t_1 and t_2 dimensions, respectively. Overlaid color circles show predicted intra-residue correlations, which were determined based on solution NMR shifts (Supporting Information, Table S1). One-bond correlations are shown in blue, except for those between C_α and C_β in helix (shown in red and labeled with the residue number). Two- and three-bond correlations are shown in light blue.

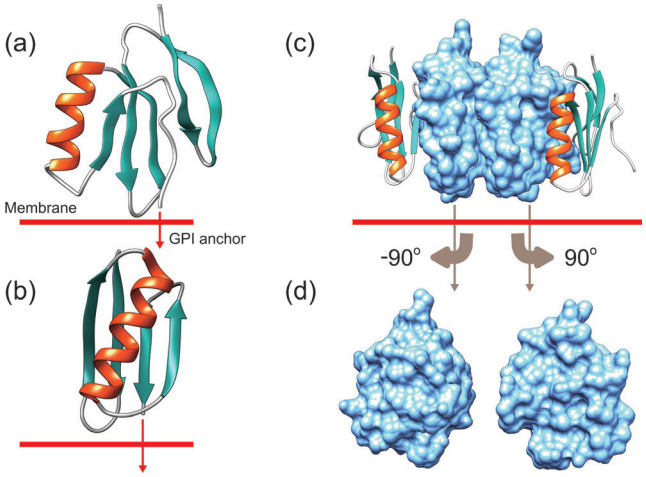

Figure 5. Ribbon models of a) Prod1 and b) GB1 on the face of the β-sheet. The α-helix and β-sheet are shown in orange and blue, respectively. The probable GPI anchor position/direction is indicated by an arrow for each protein. c) Proposed model of a Prod1 complex of four subunits shown along the membrane surface. Only the two subunits in the middle are shown by the molecular surface. d) The two subunits in the middle of the complex in (c) after opening of the dimer 90° outward.

In this study, we found that the anchoring of Prod1 induces aggregation, which further facilitates membrane adhesion. Solid-state NMR analyses revealed that the structure of Prod1 does not change upon aggregation. For a decade, the nanodisc system has been widely used in high-resolution solution NMR studies of membrane proteins.[12] However, aggregation of Prod1 is difficult to observe using a nanodisc system because only a limited number of protein molecules can be contained on a disc. Solid-state NMR was more suitable for our study because liposomes and bicelles are not limited with respect to the number of molecules that can be contained on the membrane.

One of the reasons Prod1 aggregates is because anchoring increases the Prod1 concentration and aligns the protein molecules on the membrane surface. However, GB1 did not aggregate even after anchoring. Thus, aggregation based on alignment is likely an intrinsic property of Prod1. We hypothesized that the unique shape of Prod1 plays a crucial role in aggregation. As previously reported,[3] Prod1 belongs to the three-finger protein family and has a slightly concave disc shape, as shown in Figure 5a. Figure 5c shows a theoretical model of the structure of a Prod1 aggregate, as predicted by combined knowledge-based modeling and docking simulations (Supporting Information, Figures S5 and S6).[13] The concave disc shape, which is characterized by an ordered distance and direction toward the membrane, enables Prod1 molecules to pack tightly with a complementary partner (Figure 5d). In contrast, as GB1 is a typical globular protein, the molecules cannot overlap with one another (Figure 5b). We also concluded that electrostatic interactions do not contribute significantly to Prod1 aggregation, as higher salt concentrations in the incubation buffer did not inhibit aggregation. Although hydrophobic interactions between Prod1 molecules were insufficient to promote aggregation in solution, such weak interactions would be sufficient to assist aggregation on the membrane surface due to the tight packing resulting from alignment of the Prod1 molecules.

In conclusion, we found that membrane anchoring facilitates the aggregation of Prod1 (cis interaction) and further induces a trans interaction between anchored Prod1 molecules tethered on opposing membranes. Because of this trans interaction, Prod1 can act as an adhesion protein in newt blastemal cells. It was recently reported that Prod1 acts as a cell-surface receptor for newt anterior gradient protein and transduces the signal for blastemal cell proliferation.[14] We herein propose an additional role for Prod1 as a cell-adhesion auxiliary based on its physicochemical properties. Such an ingenious mechanism of the GPI-anchor in the newt Prod1 could not have been elucidated if anchorless Prod1 were used. The semi-synthetic model system of lipid-anchored Prod1 used in the present study enabled us to investigate the molecular interaction between anchored Prod1 and opposing membranes. Further biological studies of the adhesive properties of Prod1 will hopefully provide novel clues for elucidating the details of the newt limb regeneration process. This system could be utilized in future investigations of the role of GPI-anchored proteins in regulating a diverse array of biological phenomena.

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Conflict of interest

The authors declare no conflict of interest.

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