NMR study of p47phox PX domain in the assembly and activity of phagocyte NADPH oxidase　　　　　NADPH

　　p47phox PX　　　　　　　　　　NMR

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The phagocyte NADPH oxidase is a multicomponent enzyme, indispensable for innate immunity as it produces reactive oxygen species (ROS) against invading pathogens (**Fig.1**). Substrate of the enzyme is the NADPH.　It provides the electrons for the reduction of O_2 into O_2^-, which is rapidly transformed to ROS. The NADPH binding site on NADPH oxidase remains elusive.

NADPH oxidase consists of cytosolic (p47^{phox}, p40^{phox}, p67^{phox}, and small GTPase Rac1 or Rac2) and membrane-integrated components (gp91^{phox}, p22^{phox}). In resting cells the components are separated between cytosol and membrane. Upon activation, the cytosolic components are recruited to membrane and interact with the membrane-integrated components to form a functional complex. The p47^{phox} PX domain plays a pivotal role in this recruitment process.

PX domain is a functional module that targets membranes through specific interaction with phosphoinositides. The p47^{phox} PX domain preferably binds PI(3,4)P$_2$. In activated phagocytes, the appearance of PI(3,4)P$_2$ on the membrane coupled with a conformational change of p47^{phox} that unmasks its PX domain, signals the translocation of p47^{phox} towards the membrane.

I report here 1) a novel approach for the *in vitro* study of membrane-interacting proteins based on Transferred Cross Saturation (TCS) method and the use of liposomes as membrane mimic environment, 2) the identification of the membrane embedded PI(3,4)P$_2$ interacting interface on p47^{phox} PX domain by a combination of TCS and Paramagnetic Relaxation Enhancement (PRE) experiment, and 3) evidence that p47^{phox} PX the formation of NADPH binding site on th
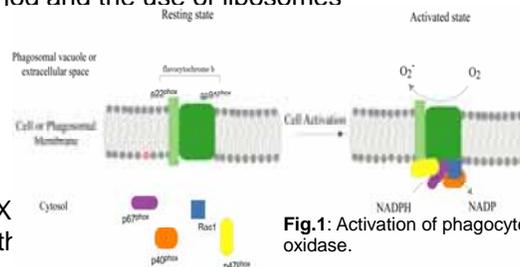
Fig.1: Activation of phagocyte NADPH oxidase.

complex.

Results and discussion

Under physiological conditions, phosphoinositides interact with effector proteins in the context of lipid bilayers and therefore the consideration of membrane-mimicking conditions for the *in vitro* study of these systems is of utmost importance. Liposomes represent the best model for biomembranes. However, their huge size hampers NMR analysis.

TCS is a novel NMR technique developed in our laboratory. It elegantly allows the amino-acid resolution power of NMR analysis to be employed for the study of protein-involved interacting systems with very large total size.   The application of TCS method in the case of a protein interacting with membrane–embedded phosphoinositide is outlined in **Fig.2** (upper panel).

Fig.2: **upper panel**: (left) Application of TCS method to a liposome system. Selective RF pulse saturates the protons in the lipid components of the liposomes, without affecting the uniformly deuterated protein. The saturation is cross-transferred to the protein in the bound state, and in the case of fast exchange it is 'carried over' to the free state. Saturation is recorded as the intensity loss of cross-peaks on the HSQC spectrum of the free protein, exclusively shown by amide protons within the interacting
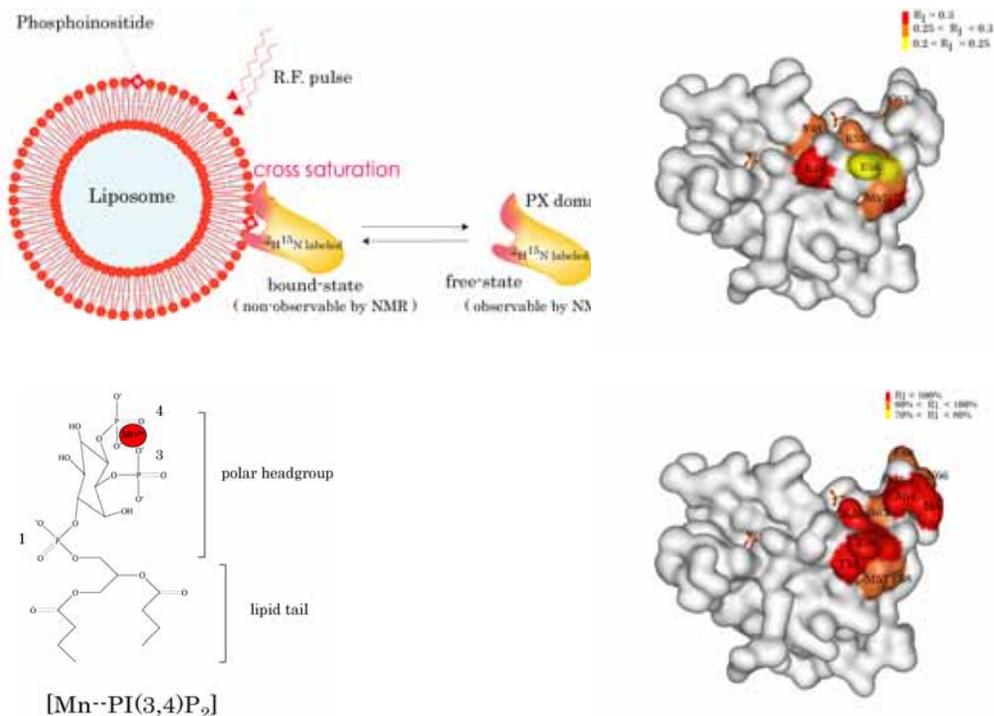
interface. (right) Results of TCS experiment.   **lower panel**: (left) PRE experiment based on the formation of [Mn—PI(3,4)P2] complex. (right) Results of PRE experiment.

1.The membrane embedded C_{16}-PI(3,4)P_2 interacting interface on p47^{phox} PX domain revealed by TCS experiment.

In order to identify the membrane interacting interface on p47^{phox} PX domain for the membrane embedded PI(3,4)P_2, TCS experiment was conducted. Uniformly labeled ^{2}H, ^{15}N p47^{phox} PX was interacted with LUV (large unilamellar vesicles) with an average diameter of 100 nm and composition of POPC : POPE : PI(3,4)P_2 = 79.5 : 20 : 0.5. Vesicles were prepared by extrusion. The experiment was conducted in ^{2}H-MES, ^{2}H-DTT, 90%D_2O, pH 5.5 at 25°C under the mode of simultaneous saturation of the protons in the lipid tails and polar headgroups of liposome components. Selective saturation of liposome components led to intensity reduction for specific signals on the HSQC spectrum of p47^{phox} PX. E49, F58, and I71 showed strong (RI > 0.3), M27,

Y48, K55, M57, and G63 showed moderate (0.3 > RI > 0.25) while H8, E15, E56, N69, and I72 displayed weak intensity reduction (0.25 > RI > 0.2). Mapping of these signals on the surface of the crystal structure of p47^{phox} PX revealed the area that contacts the C_{16}-PI(3,4)P_2 containing liposomes (**Fig.2** upper panel). In an identical experiment using control liposomes (liposomes without PI(3,4)P_2) none of the signals on the surface of p47^{phox} PX displayed intensity reduction.

2.The binding pocket of PI(3,4)P_2 headgroup on p47^{phox} PX domain revealed by Mn^{2+}-induced PRE experiment

In order to determine the binding site of PI(3,4)P_2 polar head within the membrane embedded C_{16}-PI(3,4)P_2 interacting interface, we designed an experiment based on the PRE effect of Mn^{2+} adsorbed in the soluble analogue C_4-PI(3,4)P_2. Uniformly labeled ^{15}N-p47^{phox} PX domain was interacted with C_4-PI(3,4)P_2 in 5mM MES, 5mM DTT, pH 6.4 in the presence of 0.5 mM $MnCl_2$. The HSQC spectrum of PX domain in its bound state with C_4-PI(3,4)P_2 revealed Mn^{2+}-induced PRE effect for specific signals of the protein. Their mapping on the surface of the crystal structure of p47^{phox} PX is shown in **Fig 2** (lower panel). Because of the distance-dependence mode of Mn^{2+} derived PRE, residues with 100% intensity reduction, namely T53, K55, E56 on one side and G63, A64, and I65 on the other side should form the walls of the PI(3,4)P_2 binding pocket. Altogether, the above data suggest that the PI(3,4)P_2 interacts with p47^{phox}PX in an area different from that already reported as PI(3,4)P_2 binding site. It overlaps well with the site where one of the two bound sulphates were detected in the crystal structure of the protein and it is very close to the proline rich area that is responsible for the masking of PX domain in the close conformation of p47^{phox}.

3.p47^{phox}PX domain interacts with NADPH with a *Kd* similar to that reported for the fully assembled NADPH oxidase complex

Upon addition of NADPH, specific signals on the HSQC spectrum of ^{15}N-p47^{phox} PX showed chemical shift perturbation. Mapping of these signals on the crystal structure of the protein is shown in **Fig.3**.

Residues with strong, moderate and weak normalized chemical shift change are colored red, orange and yellow respectively. Based on a NMR titration analysis, *Kd* for the interaction of NADPH with PX domain was estimated to be 69.1 uM. The reported *Kd* for the binding of NADPH on the NADPH oxidase complex is 60 uM.
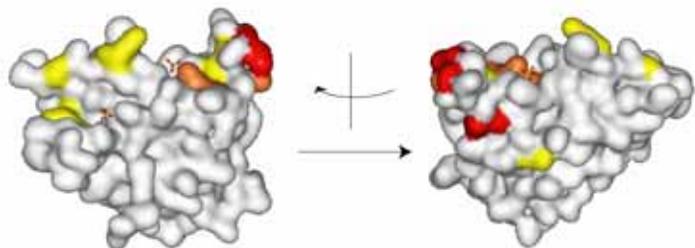
Fig.3: Chemical shift perturbation on p47^{phox} PX domain caused by NADPH.

Conclusions

In the present work, the membrane-embedded C_{16}-PI(3,4)P_2 interacting interface on p47^{phox} PX domain was successfully determined by a combination of TCS and PRE experiment using liposomes as membrane-mimicking environment. This is in contrast to conventional approaches where the presence of membrane is either ignored (use of soluble analogues of ligands) or reproduced by using micelles. The NMR method proposed here should be of great utility for the study of phosphoinositide-effector modules like PX, PH, ENTH, and C2 domain.

In addition, the interaction of p47^{phox} PX domain with the substrate NADPH, suggest that PX domain might play a dual role: it contributes to migration of the cytosolic components during assembly of NADPH oxidase and in a subsequent stage, it participates in the formation of NADPH binding site on the enzymic complex.

Reference

Pavlos Stampoulis, Hiroaki Terasawa, Hideki Sumimoto & Ichio Shimada. "Structural basis of the membrane-embedded C_{16}-PI(3,4)P_2 recognition mode of p47^{phox} phox homology domain by NMR" *submitted.*