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Activation of Phospholipase D2 through Phosphorylation of Tyrosine-470 in Antigen-stimulated Mast Cells

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The mechanism of activation of phospholipase D2 (PLD2) remains undefined although mechanisms have been described for the activation of PLD1. By expression of mutated forms of haemaglutinnin-tagged PLD2 in a mast cell (RBL-2H3) line, we show that PLD2 is phosphorylated at tyrosines -11, -14, and -470 and that tyrosine-470 is critical for activation of PLD2 by antigen. Studies were performed with mutated-DNA constructs for haemaglutinnin-tagged PLD2 in which codons for tyrosine -11, -14, -165, and -470 were mutated to phenylalanine either individually or collectively. Transient expression of these constructs showed that mutation of tyrosine -11, -14, -470, or all tyrosines (all-mutated PLD2) suppressed antigen-induced tyrosine phosphorylation of PLD2 but only the tyrosine-470 mutant failed to be activated by antigen as assessed by *in vitro* assay of immunoprepitated PLD2 or by assay of PLD in intact cells. The critical role of tyrosine-470 was confirmed in studies with add-back mutants (phenylalanine back to tyrosine) of the all-mutated PLD. The findings provide the first description of a mechanism of activation of PLD2 in a physiological setting.

Key words – PLD2; Tyrosine Phosphorylation; Mast cells

Phospholipase D (PLD) is activated via receptors in a wide variety of cells, including the mast cell, where it is thought to regulate intracellular signaling processes and cell functions such as membrane trafficking and cytoskeletal organization[1-3]. PLD catalyzes the hydrolysis of phosphatidylcholine to form phosphatidic acid which is rapidly converted in turn to lysophosphatidic acid and diacylglcerol all of which are potential messenger molecules. In the presence of relatively low concentrations of primary alcohols production of phosphatidic acid is diverted to metabolically inert phosphatidylalcohols by transphosphatidylation, a reaction unique to PLD and one that is utilized in the assay PLD *in vivo*[4]. Two forms of PLD have been cloned, PLD1 (as variants a and b) and PLD2[5,6]. PLD1 is activated *in vitro* by small GTPases and protein kinase Cα in the presence of phosphatidylinositol 4,5-bisphosphate[5,7-10]. There is also evidence that PLD1 can be regulated *in vivo* by Rho kinase[11], Ca^{2+}/calmodulin-dependent (CAM) kinase II[12], and PKC in a catalytically- dependent and independent manner[5,13,14]. PLD2, in contrast, is activated *in vitro* by phosphatidylinositol 4,5-bisphosphate alone and this activity is minimally affected by the small GTPases or protein kinase Cα[6,15,16]. However, the mechanisms regulating PLD2 activity *in vivo* are unclear. In addition to the above mechanisms, there are reports of tyrosine phosphorylation of PLD1[17,18] and PLD2[19,20] as well as pharmacological indications that tyrosine phosphorylation may regulate PLD activity[18,20-22]. In two of these reports, PLD2 was shown to associate with, and be phosphorylated by, the tyrosine kinase receptor for EGF[19] and Src kinase in oxidant stimulated cells[23]. Nevertheless, the role of such phosphorylation is uncertain. While tyrosine-11 was identified as the specific residue phosphorylated in PLD2, mutation of this site enhanced basal PLD2 activity but had no effect on the magnitude of the PLD2 response to EGF[19].

PLD is thought to play an essential role in mast cell function[24-26]. PLD is activated in isolated mast cells[27] and cultured mast cell lines[24,28,29] by a variety of stimulants including antigen which acts via the IgE receptor, FcεRI. Crosslinking of the IgE/FcεRI complex with antigen results in the recruitment and activation of several tyrosine kinases. To date, the RBL-2H3 cell line has been used exclusively to study the function of the individual PLD isoforms in mast cells. This cell line expresses PLD1b and PLD2[30]. Studies with transiently expressed forms of both PLDs in RBL-2H3 cells indicate that PLD1b and PLD2 are localized on respectively, granule membranes and the plasma membrane[25,30], and that both isoforms are activated upon antigen stimulation[31]. The mechanisms of activa-

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tion of these PLDs by antigen are unknown however the location of PLD2 at the plasma membrane makes this isoform particularly accessible to FcεRI-associated tyrosine kinases.

We report here that expressed PLD2 but not PLD1b is tyrosine phosphorylated following antigen stimulation of RBL-2H3 cells. Using mutational analysis we have identified several tyrosine phosphorylation sites of which tyrosine-470 is essential for PLD2 activation.

EXPERIMENTAL PROCEDURES

Materials-These were purchased from the following sources: L-α-phosphatidyl-D-myo-inositol 4,5-bisphosphate (PI 4,5-bisphosphate) from Boehringer-Mannheim Biologicals (Indianapolis, IN); L-α-dipalmitoylphosphatidylcholine and DNP-specific mouse IgE from Sigma (St. Louis, MO); phosphatidylethanolamine from Avanti Polar Lipids (Alabaster, Al); [*choline-methyl*-^{3}H]dipalmitoylphosphatidylcholine (50 Ci/mmol) from DuPont-NEN (Boston, MA); cell culture reagents from GIBCO/Life Technologies, Inc. (Rockville, MD); Tris-glycine polyacrylamide gels from Novex (San Diego, CA).

Transient transfection of cells with HA-PLDs-Plasmids for HA-PLDs were kindly supplied by Dr. Michael A. Frohman (Institute for Cell and Developmental Biology, State University of New York, Stony Brook, New York, 11794-8651). Mutations were made from HA-PLD2 using the site-directed mutagenesis kit (QuickChange, Stratagene, USA) with the following primers: PLD2 Y11F, 5′-GAAGAACCTCTTTCCCTTTGGGGACTATCTGAAC-3′; PLD2 Y14F, 5′-CTGGCTGGAGTTCAGAAAGTCCCCATAGGGAAAG-3′; PLD2 Y165F, 5′-GCCAGCAAACAGAAATTCTTGGAAAATTACCTC- 3′; PLD2 Y470F, 5′-CAGGTCAGTCAGTCGGAATTGCACGTCATCCCAG-3′. RBL-2H3 cells were grown as monolayers in minimal essential medium with Earle's salts, supplemented with glutamine, antibiotics, and 15% fetal bovine serum[32,32]. Cells were transiently transfected with each DNA preparation (25 μg/2×10^7 cells) by electroporation (Biorad Gene Pulser^{tm}, 960 μF, 250 V). Successful transfection was confirmed by Western blotting. Cells were used within 48 h of transfection.

Cell stimulation, immunoprecipitation of HA-PLDs, and gel electrophoresis - The PLD-transfected cells (~0.4×10^6 cells/10 cm Petri dishes) were washed with fresh growth medium 4 hs after transfection and incubated with 50 ng/ml IgE for 3 h. The cells were washed and medium replaced with a PIPES-buffered medium (25 mM PIPES, pH7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum). Cells were then stimulated with 25 ng/ml DNP-BSA for 3 min or 15 min as indicated, chilled with ice to terminate the stimulation, and then washed twice with ice-cold PBS. Cells were lysed in 0.5 ml with ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail tablet). Lysates were kept on ice for 30 min and then centrifuged 15,000×g for 15 min at 4℃. The supernatant fraction was precleared by addition of 50 μl Protein G-Agarose. After gentle shaking for 1 h, the mixture was centrifuged and samples of the supernatant fraction of equal protein content were used for immunoprecipitations. HA-PLDs were immunoprecipitated by overnight incubation (at 4℃ with gentle shaking) with agarose-conjugated anti-HA antibody. The agarose was washed five times with a washing-buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Nonidet p-40, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail tablet) and dissolved in 2x Laemmli buffer[33]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Scheicher and Schuell, BA85). The immunoreactive proteins were detected by use of horse-radish peroxidase-coupled secondary antibodies and Enhanced Chemiluminescence according to the manufacturer's instructions. (Amersham Pharmacia Biotech).

Assay of Immunoprecipitate of PLD2- PLD activity was assayed by measurement of [^{3}H]choline release from the PLD substrate, [*choline-methyl*-^{3}H]dipalmitoylphosphatidylcholine, exactly as described by Massenburg *et al.*[34,34]. Immunoprecipitates were dissolved in 100 μl buffer A (50 mM HEPES, pH 7.5, 3 mM EGTA, 80 mM KCl, 2.5 mM $MgCl_2$, and 300 nM calcium) and incubated with 25 μl of a phospholipid vesicle preparation that contained 140,000 dpm of [*choline-methyl*-^{3}H]dipalmitoylphosphatidyl- choline, dipalmitoylphosphatidylcholine, phosphatidylinositol 4,5-bisphosphate, and phosphatidylethanolamine[34]. The mixture was incubated with shaking at 37 ℃ for 1 h before addition of 1 ml of chloroform/methanol/concentrated HCl, 50:50:0.3 (vol/vol), and 0.35 ml of 1 M HCl/5 mM EGTA. The upper aqueous phase was assayed for [^{3}H]choline by liquid scintillation counting.

Measurement of PLD activity in intact cells by the trans-phosphatidylation assay- PLD-transfected RBL-2H3 cells were incubated for three hours with 50 ng/ml IgE in complete growth medium in 12-well plates (~3.5×10^5 cells/well). [^{3}H] Myristic acid, 2 μCi/ml, was added for the final 90 min of incubation. Cells were then incubated in PIPES-buffered medium[30] in the presence of 1% ethanol for 10 min before stimulation with 25 ng/ml DNP-BSA for 3 min. [^{3}H] Phosphatidylethanol was assayed by minor modifications of previously described procedures[35]. The reaction was terminated by addition 1.5 ml of a mixture of chloroform: methanol: 4N HCl (100:200:2, v/v/v) to form a single phase that was subsequently separated into two phases by addition of 0.5 ml of chloroform that contained of unlabeled phosphatidic acid and phosphatidylethanol (60 μg of each) as well as 0.5 ml of 0.1N HCl. The phospholipids in the lower chloroform phase were separated by thin layer chromatography for the assay of [^{3}H]phosphatidylethanol by liquid scintillation counting as described previously[35].

RESULTS AND DISCUSSION

*Tyrosine phosphorylation of PLD2 and identification of sites of phosphorylation-*Studies were conducted with RBL-2H3 cells made to express HA-PLD1 or HA-PLD2. Immunoprecipitation of HA-tagged PLDs and immunoblotting with antibodies against HA and phosphotyrosine revealed that HA-PLD2 but not HA-PLD1 was tyrosine phopshorylated and the extent of this phosphorylation was increased upon antigen stimulation (Fig. 1A) in a time dependent manner (Fig. 1B and C). The increased phosphorylation was evident within 1.5 min and reached a maximum by 15 min.

Examination of the amino acid sequences of both PLDs indicated four tyrosines that were unique to PLD2 namely, Y11, Y14, Y165, and Y470, as has been reported by other workers[19]. We next investigated the effects of mutation of these individual sites (Y to F) and of all four sites simultaneously (Y11/14/165/470F) on PLD2 phosphorylation following the expression of these mutants in RBL-2H3 cells. Phosphorylation was impaired in the Y11F, Y14F, and Y470F mutants and virtually abolished in the Y11/14/165/470F mutated PLD2 (Fig. 2). These mutations appeared to impair both basal (i.e., in non-stimulated cells) and antigen-stimulated phosphorylation. These results suggested that Y11, Y14 and Y470 were the sites of phosphorylation and that sites other than these were minimally or not phosphorylated.

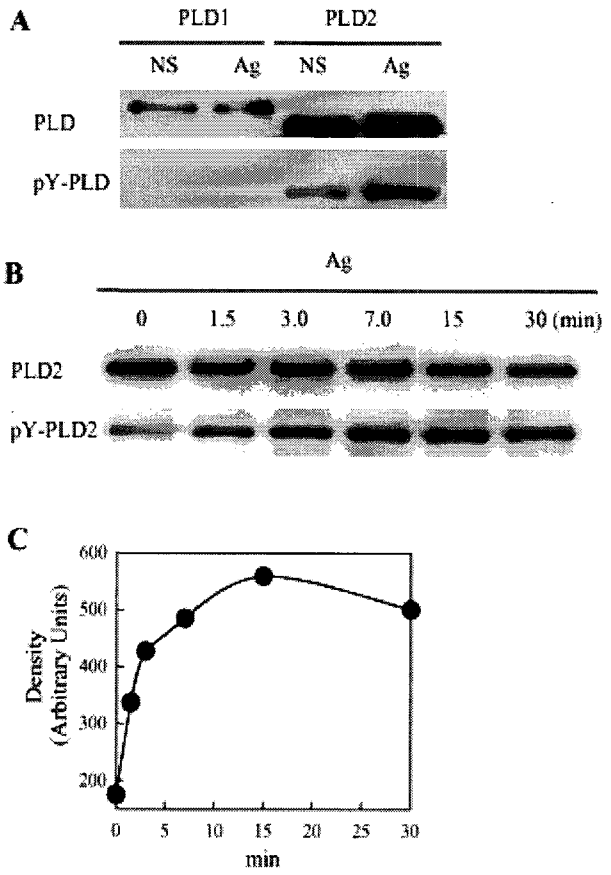

Fig. 1. HA-PLD2, but not HA-PLD1, is tyrosine phosphorylated in antigen-stimulated cells. RBL-2H3 cells transiently transfected with HA-PLD1 or HA-PLD2 DNA-constructs and primed with DNP-specific IgE before stimulation with antigen (25 ng/ml DNP-BSA) for 3 min (*A*) or as indicated (*B* and *C*). The PLDs were immunoprecipitated with anti-HA antibody and separated by SDS-PAGE for detection with anti-HA and anti-phosphotyrosine antibodies. Representative immunoblots are shown in Panels A and B and the average values for density of bands for phosphorylated PLD2 from three experiments are shown in *C*. Key: NS, non-stimulated; Ag, antigen stimulated; pY-PLD, tyrosine phosphorylated PLD and PLD2.

*Tyrosine-470 is critical for PLD2 activation-*Measurement of PLD activity by the [^{3}H]choline-release assay *in vitro* showed that the intrinsic activity of immunoprecipitated HA-PLD2 increased following antigen stimulation of cells made to express *wt* HA-PLD2 (Fig. 3A). The increase was not apparent when cells were made to express the Y470F mutant or the Y11/14/165/470F mutant. All other single point mutations were without effect (Fig. 3B). The levels of PLD activity in the immunoprecipitated Y470F and Y11/14/165/470F mutated PLDs were comparable to the activity of immunoprecipitated *wt* HA-PLD from unstimulated cells. The

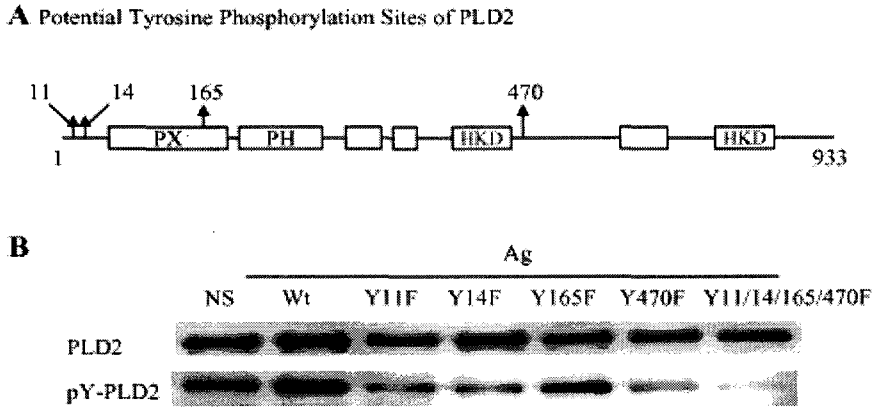

Fig. 2. Mutation of three tyrosines sites that are unique to PLD2 impair phosphorylation of PLD2. Four tyrosine residues in PLD2 that have no corresponding tyrosines in PLD1 are shown in *A*. DNA constructs of HA-PLD2 with mutations of the individual tyrosines (tyrosine to phenylalanine) or with mutations of all four tyrosines (Y11/14/165/470Y) as well as HA-PLD2 (*wt*) were transiently expressed in RBL-2H3 cells and experiments were performed as described for Figure 1. Cells were stimulated for 3 min with antigen (Ag) or left unstimulated (NS) for immunoprecipitation and immunoblotting for detection of HA-PLD2 and tyrosine-phosphorylated HA-PLD (pY-PLD2) (*B*). The blots are representative of three such experiments.

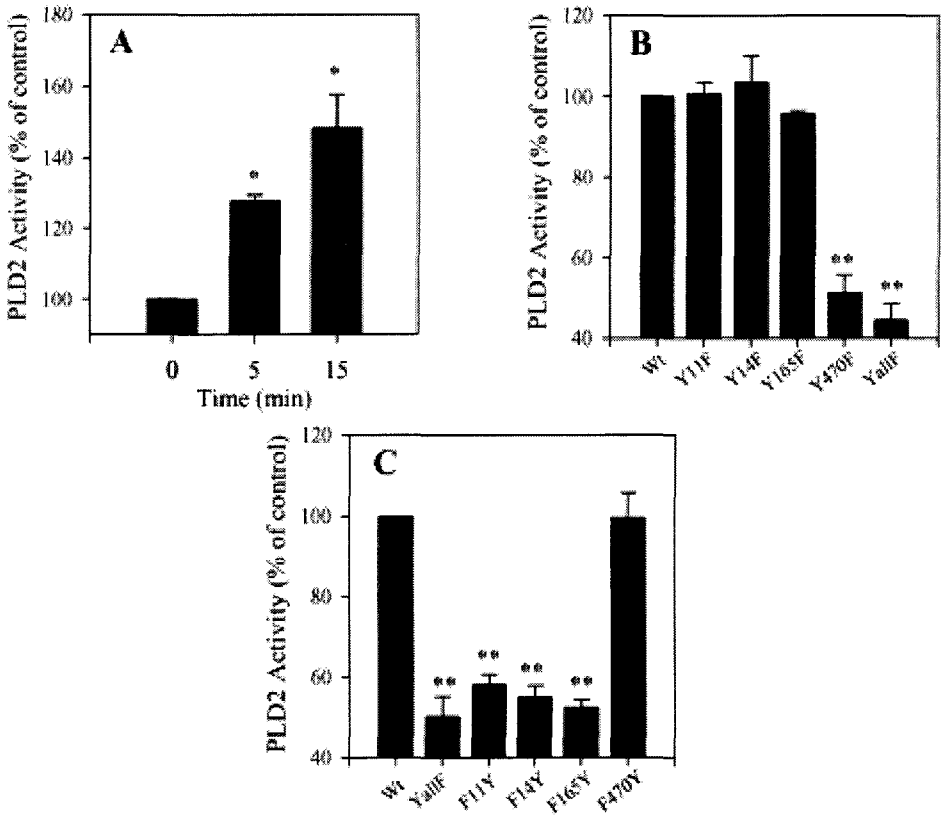

Fig. 3. Tyrosine-470 is critical for PLD2 activation by antigen as measured by *in vitro* assay. Experiments were performed with point or all site mutated-DNA constructs described for Figure 2 and with constructs prepared by add-back mutations (phenylalanine back to tyrosine) of each site of the Y11/14/165/470F construct (YallF). *Wt* HA-PLD (*A*), the mutated HA-PLDs (*B*), and add-back mutated HA-PLD (*C*) were expressed in RBL-2H3 cells and stimulated with antigen as described for Figure 1 for the indicated times (*A*) or 15 min (*B* and *C*). The expressed HA-PLDs were immunoprecipitated and assayed for PLD activity by the *in vitro* [^{3}H]choline-release assay. Values shown are the mean ±SEM of values from three separate experiments and are expressed as percent of activity at zero time (14,420±610 dpm [^{3}H]choline released) (*A*) or of stimulated activity of immunoprecipitated HA-PLD (*wt*) (21,333±1375 dpm [^{3}H]choline released). Significant differences in values compared to zero time (*A*) or *wt* HA-PLD (*B* and *C*) are indicated by asterisks; *, $P<0.05$ and **, $P<0.01$.

requirement for Y470 was confirmed by individual add-back mutations of the Y11/14/165/470F mutant (Fig. 3C). Mutation of F470 to Y470 alone fully restored the ability of PLD2 to be activated by antigen. Other mutations were ineffective in restoring activity.

The requirement Y470 was apparent also by measurement of PLD activity in intact cells by the transphosphatidylation assay (Fig. 4). As with the *in vitro* assay system, expression the Y470F PLD2 mutant abrogated activation of PLD by antigen whereas the add-back F470Y mutant completely restored such activation.

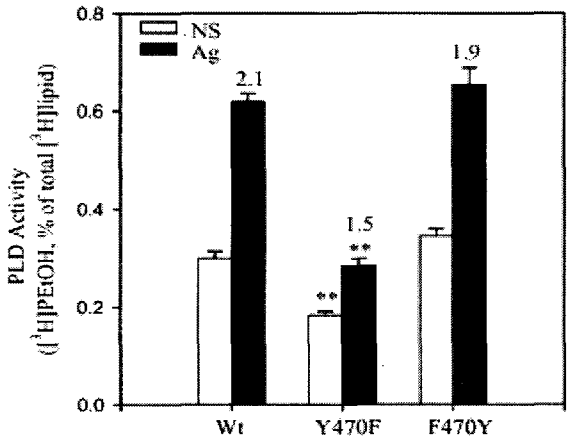

Fig. 4. Tyrosine-470 is critical for PLD2 activation by antigen *in vivo*. DNA constructs of *wt* HA-PLD2, the HA-PLD2 mutant (Y470F), and add-back mutant (F470Y of the YallF mutant of HA-PLD2) were transiently expressed in RBL-2H3 cells. The cells were labeled with [^{3}H]myristate before stimulating with antigen (Ag) or not (NS) for 3 min in the presence of 1% ethanol for measurement of [^{3}H]phosphatidylethanol ([^{3}H]PEtOH) to assess *in vivo* PLD activity (the transphosphatidylation assay). Values shown are the mean ±SEM of values from three separate experiments and are expressed as percent of total radioactivity recovered in the lipid fraction. Numeric values indicate fold increase in activity and ** indicates significant difference (P<0.01) from corresponding values for *wt* HA-PLD2.

Concluding comments- The present results show that PLD2 is phosphorylated predominantly, if not exclusively, on Y11, Y14, and Y470 in RBL-2H3 cells. Also, this phosphorylation is increased upon antigen stimulation as is the PLD activity when measured *in vitro* with immunoprecipitated PLD2 or *in vivo* in the intact cell. Furthermore, phosphorylation of Y470 is critical for PLD activation by antigen.

The activation of signaling pathways in mast cells by antigen is dependent on tyrosine kinases such as Lyn and Syk[36] and on the interaction of the IgE receptor with these kinases in lipid rafts[37]. Either of these kinases are candidates for phosphorylating PLD2 and preliminary observations indicate that disruption of lipid rafts to indicate a possible link to FcεRI.

Of relevance to our studies is the report that PLD2 associates with the EGF tyrosine kinase receptor and is phosphorylated at Y11 upon activation of the receptor[19]. Mutation of this residue to phenylalanine prevented phosphorylation but did not impair or enhance the increase in PLD activity in response to epidermal growth factor as demonstrated by co-expression of PLDs and EGF receptor into HEK293 cells. Other PLD2 mutations namely, Y14F, Y165F, and Y470F did not impair phosphorylation to the same extent and were not investigated further. These and our findings suggest that PLD2 can be tyrosine phosphorylated at one or multiple sites by a receptor tyrosine kinase or by a receptor-activated tyrosine kinase. The role of phosphorylation of tyrosine residues Y11 and Y14 is unclear, because these do not appear to be activating phosphorylations, although ancillary functions such as binding to SH2-containing signaling molecules cannot be ruled out. Interestingly, Y470 lies in close proximity to one HKD motif, and as shown here its phosphorylation appears to be a critical, and the first described, mechanism for the activation of PLD2 *in vivo*.

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PLD1 활성화 기전은 여러 보고가 있으나 PLD2 활성화에 대한 기전은 아직 연구의 대상이다. RBL-2H3 비만세포에서 HA-PLD2의 인산화 가능한 타이로신 잔기를 점돌연변이 시킨 DNA 플라즈미드를 이용하여 11번, 14번, 470번의 타이로신이 항원자극에 의해 인산화 됨을 알아냈고 특히 470번 타이로신의 인산화가 PLD2 활성화에 중요하다는 결과를 얻었다.