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Identification of Ser/Thr phosphorylation sites in the C2-domain of Phospholipase C γ 2 (PLC γ 2) using TRPM7-kinase

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Abstract

PLC-isozymes are central elements of cellular signalling downstream of numerous receptors. PLC γ 2 is a pivotal component of B cell receptor (BCR) signalling. The regulation of PLC γ 2-dependent signalling functions by Tyr-phosphorylation is well characterized, however, the potential role of Ser/Thr phosphorylation events remains undefined. TRPM7 is the fusion of a Ser/Thr kinase with an ion channel, and an essential component of Mg²⁺-homeostasis regulation. Although the interaction between the C2 domain of several phospholipase C (PLC) isozyms and TRPM7 is well established, previous studies have focused on the effect of PLC-activity on TRPM7. Here, we investigated whether Ser/Thr phosphorylation sites in the C2 domain of PLC γ 2 could be identified using TRPM7-kinase. We show that TRPM7-kinase phosphorylates PLC γ 2 in its C2-domain at position Ser1164 and in the linker region preceding the C2-domain at position Thr1045. Using a complementation approach in PLC γ 2^{-/-} DT40 cells, we found that the PLC γ 2-S1164A mutant fully restores BCR mediated Ca²⁺-responses under standard growth conditions. However, under hypomagnesian conditions, PLC γ 2-S1164A fails to reach Ca²⁺-levels seen in cells expressing PLC γ 2 wildtype. These results suggest that Mg²⁺-sensitivity of the BCR signalling pathway may be regulated by Ser/Thr phosphorylation of PLC γ 2.

Keywords

PLC γ 2; Ser/Thr phosphorylation TRPM7; Hypomagnesia; B Cell Receptor signalling

1. INTRODUCTION

PLC enzymes are crucial signalling elements that are recruited to the membrane and activated following the ligation of numerous cellular receptors. PLC proteins convert the membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to the second messengers

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Diacylglycerol (DAG), a Protein Kinase C activator, and the Ca^{2+} -store mobilizing agent 1,4,5- trisphosphate (InsP_3) [1]. The enzymatic activity and membrane recruitment of PLCs are tightly regulated, and the modular architecture of PLC proteins underscores the complex interdomain and protein-protein interactions involved in ensuring their adequate activation in response to receptor stimulation [2]. Beyond their catalytic regions, PLCs additionally contain various combinations of PH-domains (Pleckstrin Homology), Ca^{2+} -binding EF-hands, SH2/SH3 protein-protein interaction domains, as well as C2 domains that often play the role of Ca^{2+} -sensing phospholipid binding modules [3]. Phosphorylation of specific residues is essential to controlling PLC activity, and the contribution of multiple families of Tyr-kinases in this process is well studied [2, 4]. The influence of Ser/Thr phosphorylation events on PLC-function is however only weakly characterized. In a yeast two-hybrid screen, the C2 domain of phospholipase C β -1 ($\text{PLC}\beta$ 1) was found to interact with the TRPM7 Ser/Thr-kinase [5]. This interaction was confirmed in protein-pulldown assays, which additionally demonstrated that $\text{PLC}\beta$ 2, β 3, and γ 1 also interact with TRPM7-kinase [6].

The vertebrate versions of TRPM7 and its closest homologue TRPM6 are the only known examples of ion channels covalently linked to a kinase domain. Both channels are essential regulators of Mg^{2+} -homeostasis. TRPM6-deficient patients are hypomagnesic and suffer from debilitating seizures, but can live a normal life if supplemented with high levels of Mg^{2+} [7, 8]. Similarly, TRPM7^{-/-} DT40 B cells and murine embryonic stem cells can be rescued from growth arrest and cell death when cultured in media containing elevated Mg^{2+} levels [9, 10].

Under physiological conditions, TRPM7 is a divalent cation selective channel inhibited by intracellular Mg^{2+} or MgATP [11]. This Mg^{2+} -sensitive gating does not require TRPM7-kinase activity [10, 12]. As there are numerous examples of ion channels regulated *in trans* by phosphorylation events, the presence of a kinase domain in TRPM7 raises the question whether beyond the well-documented autophosphorylation of TRPM7 [10, 13, 14], its kinase domain can also function as a signalling module through phosphorylation of exogenous substrates. In the past years, several substrates of TRPM7-kinase were described, including Annexin I and Myosin IIA [15, 16]. TRPM7-kinase is also involved in adjusting the activity of the eukaryotic elongation factor eEF2 in accordance to the environmental availability of Mg^{2+} via phosphorylation of its kinase eEF2-k [17].

We propose that the implication of the observed association between TRPM7-kinase and PLCs could thus be two-fold: i) TRPM7 activity might be influenced by PLC and its substrate and/or products, which might not necessarily require the direct interaction between TRPM7 and PLCs. Sensitivity to PIP_2 has been ascribed to numerous ion channels, including other TRP family members [18, 19]. Several studies have focused on the effect of PIP_2 on TRPM7, some finding TRPM7 to be inhibited by PIP_2 -hydrolysis, and others reporting the opposite or no effect [6, 20, 21]. ii) PLC enzymes could be substrates of the TRPM7 Ser/Thr kinase, and TRPM7 could be an unsuspected modulator of PLC activity. In B lymphocytes, $\text{PLC}\gamma$ 2 is at the heart of BCR signalling. $\text{PLC}\gamma$ 2-deficient mouse models exhibit defective B cell development and function, including reduced numbers of mature follicular B cells and weak responses to T-independent type 2 antigens [22, 23].

Acute activation events following BCR ligation typically involve phosphorylation of selected tyrosine residues, which are well-characterized in PLC γ 2 [24]. From our review of the literature, little is known about the potential modulation of PLC-activity via Ser/Thr phosphorylation. Here, we hypothesized that TRPM7-kinase could be used to identify new Ser/Thr phosphorylation sites in the C2-domain of PLC γ 2. We present data indicating that TRPM7 phosphorylates PLC γ 2 at position Ser1164 in the C2-domain, and at position Thr1045 in the linker between the catalytic region and the C2 domain. Mutation of the Ser1164 position to Alanine leads to a similar Ca²⁺-response under physiological Mg²⁺-conditions, but a lower level of cytoplasmic Ca²⁺-elevation under hypomagnesian conditions. These data thus provide first clues that Ser/Thr phosphorylation of PLC γ 2 might contribute to adjusting the signalling intensity of PLC γ 2-dependent pathways according to the availability nutrients, such as the biologically essential ion Mg²⁺.

2. MATERIAL AND METHODS

2.1 Molecular biology and cell line generation

Human (h)PLC γ 2 was subcloned into pcDNA4/TO (Invitrogen), and the C2-domain of PLC γ 2 into pcDNA4/TO-NFlag. The PLC γ 2 S1164A mutation in full-length and C2-domain constructs, and the nine mutants individually replacing the Thr-residues in the C2-domain construct by Ala, were generated by PCR and verified by sequencing. TRPM7 constructs were previously described [10, 17]. PLC γ 2^{-/-} DT40 cells were stably transfected by electroporation with hPLC γ 2 wildtype (WT) or PLC γ 2 S1164A constructs.

Cell culture—DT40 cells [25] were maintained in RPMI, 10% FBS, 1% chicken serum. For Mg²⁺-deprivation experiments cells were grown in complete chemically defined HyQ CCM1 media (Hyclone) containing 1% chicken serum, with 0 or 1 mM MgCl₂. HEK293 cells (Invitrogen T-REx system, and [11]) were maintained in DMEM 10% FBS, with Blasticidin S (5 μ g/ml, Invivogen). Zeocin (Invitrogen) was added for HEK293 cell lines (400 μ g/ml) or DT40 cells (1 mg/ml) stably transfected with pcDNA4/TO constructs. Protein overexpression was induced with 100–1000 ng/ml doxycycline for 24–48 h.

2.2. Immunoprecipitations, immunoblotting, and in vitro phosphorylation assays

Lysis, *in vitro* phosphorylation, SDS gel electrophoresis and immunoblotting study conditions have been described previously [17]. Immunoprecipitations were performed from HEK293 or DT40 WT cell lysates using antibodies against Flag (Sigma), HA (Sigma), PLC γ 2 (Santa Cruz) or IgG conjugated to protein G beads. Membranes were probed with these antibodies or a motif specific anti phospho-Ser antibody (RxY/FxpS) (Cell Signaling). Western Blot analysis was performed using the Li-Cor ODYSSEY Infrared Imaging System or Immobilon Western Chemiluminescent HRP Substrate (Millipore) and Super-RX Medical X-ray film (Fuji).

2.3. Ca²⁺ measurements

Cytosolic [Ca²⁺] was evaluated using stable PLC γ 2^{-/-} DT40 cell lines expressing PLC γ 2 WT or PLC γ 2 S1164A grown in chemically defined media with 0 or 1 mM MgCl₂. At 15–24 h 4 \times 10⁶ cells were loaded with 1 μ g/ml Fura-2 (Invitrogen) for 30 min at 25°C in

Ringer buffer and analyzed using a bulk assay as previously described [14]. Anti-chicken IgM mAb M4 (1.2 $\mu\text{g/ml}$) was added at 40 sec.

2.4, Calculations and statistical analysis

All experiments were repeated at least 3 times with consistent results. The area under the curve was calculated by subtracting the baseline average (the average fluorescence ratio of the first 39 sec) from each time point in the response (40 sec – 420 sec) and these values were summed. The SEM was calculated for each group and paired two-tailed t-test performed.

3. RESULTS

3.1. PLC γ 2 is phosphorylated in its C2-domain on Ser1164 by TRPM7-kinase

Biochemical studies tested the interaction of TRPM7-kinase with several PLC isozymes, and found that whereas PLC β 1, - β 2, and - β 3, and - γ 1 associate with TRPM7-kinase, PLC β 4 and - δ 1 do not [6]. Because we are interested in TRPM7 function in B lymphocytes, we first asked the question whether PLC γ 2 associates with TRPM7. The DT40 chicken DT40 B cell line shows high rate of homologous recombination allowing for the efficient targeting of genomic regions to disrupt genes of interest. It has been extensively used to study the effect of deleting specific molecules on the B cell receptor signalling pathway [26]. We found that available TRPM7-specific antibodies do not allow for detection of native TRPM7 levels in DT40s, prohibiting the verification of native PLC γ 2/TRPM7 association. We thus looked at this association in TRPM7^{-/-} DT40 B cells complemented with HA-tagged human TRPM7 we generated previously [10], as it comes closest to the physiological situation. Native chicken PLC γ 2 can be analyzed in DT40s, and we found TRPM7-currents to be only 2–3 \times WT levels in these cells (Supporting Fig. S1A), which is substantially less than typical current amplitudes observed in HEK293 cells overexpressing TRPM7 (10–50 \times) [11]. We precipitated PLC γ 2 and subsequently tested for co-association with HA-TRPM7 by Western blot (Fig. S1B). Using this experimental system, we documented that native PLC γ 2 and full-length HA-tagged TRPM7 interact in DT40 cells. Encouraged by this finding, we decided to next analyze whether PLC γ 2 is a substrate of TRPM7-kinase.

Previous studies have focused on the role of PLC enzymes as regulators of phosphoinositide metabolism on the channel activity of TRPM7. However, in contrast to other ion channels, the relationship between TRPM7 and PLC enzymes is unique as it involves the kinase domain of TRPM7. We thus hypothesized that PLC proteins interacting with TRPM7 might be substrates of TRPM7-kinase. To test this idea, we performed *in vitro* phosphorylation reactions with immunoprecipitated epitope-tagged TRPM7-kinase and the C2 domain of PLC γ 2 (schematic representation of the constructs in Fig. 1A). To detect potential Ser-phosphorylation of PLC γ 2, we used an anti-phospho-Ser motif antibody (R_xY/F_xpS). We found that PLC γ 2 was only recognized by this antibody in the presence of TRPM7-kinase WT, but not of its kinasedead counterpart (K1648R) (Fig. 1B, lanes 2 and 4). This observation shows for the first time TRPM7-kinase mediated phosphorylation of a PLC isozyme, in this case of PLC γ 2.

We next aimed at identifying which serine position(s) in the C2-domain of PLC γ 2 is phosphorylated by TRPM7. After screening the PLC γ 2-C2 protein sequence, we concluded that from the 15 serine residues present, only two, Ser1164 and Ser1265, were in a context that is similar to the recognition motif of the phospho-Ser antibody (R_xY/F_xpS). Moreover, Ser1164 and the surrounding region are conserved in chicken, murine, rat, and human PLC γ 2 sequences (Fig. 1C). We therefore introduced a mutation resulting in a serine to alanine exchange at position 1164 (S1164A) to test whether this mutation would abrogate TRPM7-dependent serine phosphorylation of PLC γ 2-C2. Our results show that indeed, following an *in vitro* phosphorylation reaction with WT TRPM7-kinase, the S1164A mutant fails to be detected by the phospho-Ser specific antibody (Fig. 1B, lane 3). Additionally, to investigate the specificity of TRPM7-mediated Ser-phosphorylation of PLC γ 2, we asked whether eEF2-kinase, a protein closely related to TRPM7-kinase, can also phosphorylate PLC γ 2. Although we found that eEF2-k was recognizing and phosphorylating its cognate substrate eEF2, it had no effect on PLC γ 2 Serphosphorylation level (Fig. S2). Our data thus indicate that TRPM7-kinase specifically phosphorylates PLC γ 2 in its C2-domain on Ser1164.

3.2. TRPM7-kinase phosphorylates PLC γ 2 on Thr1045 in the linker region connecting its catalytic domain to the C2-domain

When performing the phosphorylation experiments described in the previous section, we noticed the formation of a doublet band in the samples where PLC γ 2-C2 was phosphorylated by TRPM7-kinase. Both bands are equally detected by the anti phospho-Ser antibody, and the doublet can also be seen in the S1164A mutant (Fig. 1B), indicating that this migratory shift does not originate from the S1164 phosphorylation and may thus be caused by one or multiple additional phosphorylation sites. Because the higher PLC γ 2 band was not recognized by the antiphospho-Ser motif antibody we used, we concluded that the potential site(s) might be a Thr residue(s). To test this, we performed a phosphorylation experiment as previously, but used a phospho-Thr specific antibody to detect phosphorylation. We developed the Western blot membrane using an infrared scanner allowing for simultaneous visualization of Flag-specific stain (in green), and phospho-Thr specific signals (in red). This approach clearly demonstrated that only the upper band of the phospho-PLC γ 2 doublet is recognized both by the anti-flag and the anti phospho-Thr antibodies (co-stained in yellow, Fig. 2). Analyses of the PLC γ 2-C2 S1164A mutant additionally showed that the same migration shift and phospho-Thr specific detection can be seen, indicating that the observed Thr-phosphorylation is independent of S1164 phosphorylation. Control reactions using the kinase-dead TRPM7-KR mutant showed no Thr-phosphorylation of PLC γ 2-C2 (Fig. 2 lanes 5, 6). We next therefore aimed at further defining the Thr residues(s) phosphorylated by TRPM7-kinase.

Nine threonine residues are present in the PLC γ 2-C2 domain construct used in this study (Thr1045, Thr1062, Thr1101, Thr1102, Thr1116, Thr1121, Thr1152, and Thr1219, see also Fig. S3). Because we could not discern a particular Thr residue that would be a good candidate phosphorylation site, we generated nine mutants of PLC γ 2-C2 by site-directed PCR mutagenesis, in which each of the Threonine residues was singly mutated to Alanine. Phosphorylation experiments with TRPM7-kinase revealed that only one of the mutations,

T1045A, resulted in loss of the electrophoretic migration shift, as well as of the phospho-Thr signal (Fig. 3A). In contrast, when the same set of Thr-mutants was analyzed using the anti phospho-Ser antibody and the S1164A construct as a negative control, the phosphorylation signal was not affected (Fig. 3B). Thus, the TRPM7-mediated S1164 and T1045 phosphorylation events do not appear to be interdependent.

In sum, our results show that T1045 and S1164 represent two previously unknown phosphorylation sites in PLC γ 2 that are potential substrates of the TRPM7 channel-kinase. Whereas S1164 is located in the C2-domain itself, Thr1045 is at the very N-terminal edge of the PLC γ 2-C2 construct we designed and used here, and is described as being located in the linker region between the catalytic region and the C2 domain [27]. Because the original goal of the present study was to characterize the effect of Ser/Thr phosphorylation in the C2-domain of PLC γ 2, we focused in the following on characterizing the potential importance of Ser1164 for PLC γ 2-mediated Ca²⁺-signalling.

3.3. The PLC γ 2 mutant S1164A supports normal BCR mediated Ca²⁺-responses under physiological Mg²⁺-levels, but reduced responses under hypomagnesianic conditions

To investigate the effect of mutating Ser1164 on PLC γ 2 function in the context of BCR signalling, we chose a complementation approach in a PLC γ 2-deficient DT40 B cell line [25]. To this end, we generated DT40 B cells with stable expression of WT or S1164A full-length hPLC γ 2 in the PLC γ 2^{-/-} background. We first tested the ability of PLC γ 2 S1164A to restore Ca²⁺-responses following BCR stimulation with anti-IgM using the Ca²⁺-sensitive fluorescent dye Fura-2. Under standard growth conditions, we found no difference in the amplitude or shape of the Ca²⁺-response between PLC γ 2^{-/-} DT40 cells complemented with PLC γ 2 WT or S1164A (Fig. 3A left panel). Since TRPM7 is proposed to play the role of an environmental sensor of Mg²⁺-availability, we hypothesized that the S1164A mutation might exhibit altered signalling properties in comparison to PLC γ 2 WT under suboptimal Mg²⁺-conditions. We therefore cultured the PLC γ 2^{-/-} cells expressing either PLC γ 2 WT or S1164A in Mg²⁺-free medium for 15–25 h, and repeated the Ca²⁺-measurements following BCR activation. We found the BCR mediated Ca²⁺-response to be reduced when DT40 cells were grown under hypomagnesianic conditions (Supporting Fig. S2B, Fig. 4A and B). The amplitude of the Ca²⁺-signal in Mg²⁺-starved PLC γ 2^{-/-} cells that express the S1164A mutant was consistently even further diminished (Fig. 4A right panel, quantification in Fig. 4B). This effect was not due to a difference in protein expression levels between PLC γ 2 WT and PLC γ 2-S1164A, as the protein amounts appear well matched (Fig. 4C). Additionally, it is important to mention that the Mg²⁺-deprivation protocol the cells were exposed to did not result in altered BCR surface expression (Supporting Fig. S4B). Combined with the lack of effect of the S1164A mutation at physiological 1 mM MgCl₂ levels, these results indicate that the S1164A PLC γ 2 mutant exhibits a changed sensitivity to suboptimal Mg²⁺-conditions. These findings provide a novel molecular mechanism that may explain the relationship between insufficient availability of Mg²⁺ and the efficiency of immune responses.

4. DISCUSSION

Although the interaction between TRPM7-kinase and several PLC isozymes has been known for several years [5, 6], subsequent studies have focused on the modulation of TRPM7 channel activity by the substrate of PLC isozymes, PIP₂ [6, 20, 21]. The reverse scenario, the phosphorylation and regulation of PLC proteins by TRPM7's Ser/Thr-kinase, has to our knowledge not been previously considered. The data presented here show that PLC γ 2 can be phosphorylated by the Ser/Thr kinase domain of TRPM7, allowing us to identify two novel phosphorylation sites in PLC γ 2: The conserved Serine residue 1164, located in the C2 domain of PLC γ 2 (Fig. 1), and a Thr residue (Fig. 2), Thr 1045, located in the linker region between the catalytic region and the C2-domain of PLC γ 2 (Fig. 3). Mutating Ser1164 in hPLC γ 2 and expressing this mutant in PLC γ 2^{-/-} DT40 B cells results in a full restoration of the BCR mediated Ca²⁺-signal under standard growth conditions. However, when compared to WT PLC γ 2, the S1164A mutant can only sustain a reduced Ca²⁺-response following Mg²⁺-deprivation (Fig. 4).

PLC enzymes are crucial elements of cellular signal transduction. The modular structural features and complex regulation of these enzymes reflect the diversity of their function, from cell proliferation and survival, to immunity [2]. PLCs are mainly cytosolic, and translocate to the membrane upon receptor stimulation. Membrane recruitment is thus critically regulated. The C2 domain of PLC γ 2 contains several Ca²⁺-binding motifs, and using a complementation approach in PLC γ 2^{-/-} DT40 cells, it was shown to mediate the Ca²⁺-dependent increase in PLC γ 2 translocation to the membrane following the initial release of the Ca²⁺-stores [28]. Therefore, Ser/Thr phosphorylation in this domain might influence the intensity and sustainability of the Ca²⁺-signal downstream of BCR ligation, as we observe in the S1164A PLC γ 2 mutant. The potential modulation of PLC γ enzymes via phosphorylation on Ser/Thr residues is very poorly characterized. Although members of the PLC β subgroup have been found to be phosphorylated by various Ser/Thr kinases, including ERK [29], and CaMK II [30], from our review of the literature, no functional characterization of Ser/Thr phosphorylation events have been described in PLC γ 2.

Given the essential and ubiquitous nature of Mg²⁺ in biology, it is perhaps not surprising that B cells adjust their level of BCR responsiveness to Mg²⁺ availability. The decrease in BCR dependent Ca²⁺-influx we observe under hypomagnesian conditions is accentuated in the S1164A mutant indicating that phosphorylation of Ser1164 may have an enhancing effect on PLC γ 2- activation. However, Ser1164 phosphorylation might not be reflective of the total effect of TRPM7-mediated phosphorylation of PLC γ 2, as the remaining electrophoresis migratory shift we observe in PLC γ 2-S1164A suggested at least one additional site, which we identified as Thr1045. Also, beyond TRPM7, multiple kinases might be involved in modulating the Ser/Thr phosphorylation levels of PLC enzymes in intact cells.

It is interesting to note that beyond its role as a “maintenance ion”, Mg²⁺ is also discussed as having more specific signalling functions in lymphocytes. In B cells the elevation of free [Mg²⁺]_i follows the [Ca²⁺]_i mobilization in response to receptor activation or ionophore treatment [31]. In B cell blasts, a Mg²⁺ dependent PLC activity was discovered [32]. In a

recent study, immunodeficient patients were found to exhibit a genetic deficiency in MagT1 [33], a Mg^{2+} - transporter that we have shown to partially complement for TRPM7-deficiency in DT40s [34]. MagT1 was shown to mediate a transient Mg^{2+} -influx that is crucial for T cell activation [33]. Collectively, these findings and the data presented here suggest that responsiveness to alterations in intra- and extracellular Mg^{2+} levels are an integral part of immune receptor signalling.

Our current working model is that TRPM7 via its kinase domain is ideally poised to act as an environmental sensor of Mg^{2+} -availability to guide the cell in mounting a global response for example in situations of hypomagnesia. This could include measures to conserve Mg^{2+} by reducing the rates of translational elongation [17], but also as implied by the phenotype of the S1164A PLC γ 2 mutant, by ensuring that crucial signalling pathways maintain a sufficient level of activation. In analogy to the “two-signal” model and the role of the mTOR Ser/Thr kinase in T cells [35], Ser/Thr phosphorylation of PLC isozymes via TRPM7 and other kinases might therefore contribute to the integration of environmental cues into the activation of immune responses.

5. CONCLUSIONS

1. hPLC γ 2 interacts with hTRPM7 in DT40 B cells.
2. The Ser/Thr kinase domain of TRPM7 phosphorylates PLC γ 2 on several sites.
3. Thr1045 and Ser1164 are two novel phosphorylation sites in PLC γ 2 that were identified using TRPM7-kinase.
4. PLC γ 2^{-/-} DT40 B cells complemented with the PLC γ 2-S1164A mutant show normal BCR mediated Ca^{2+} -responses under physiological Mg^{2+} -levels, but reduced responses under hypomagnesian conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TRPM7	Transient Receptor Potential Melastatin 7
PLCγ2	Phospholipase C γ 2
WT	wildtype

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Highlights

- PLC γ 2 interacts with the Mg²⁺-sensing channel-kinase TRPM7.
- The C2-domain of PLC γ 2 is phosphorylated by TRPM7's Ser/Thr kinase.
- TRPM7-mediated PLC γ 2 phosphorylation reveals two novel phospho-sites, T1045 and S1164.
- PLC γ 2-S1164A mutation results in diminished BCR-mediated Ca²⁺-signal under low Mg²⁺.

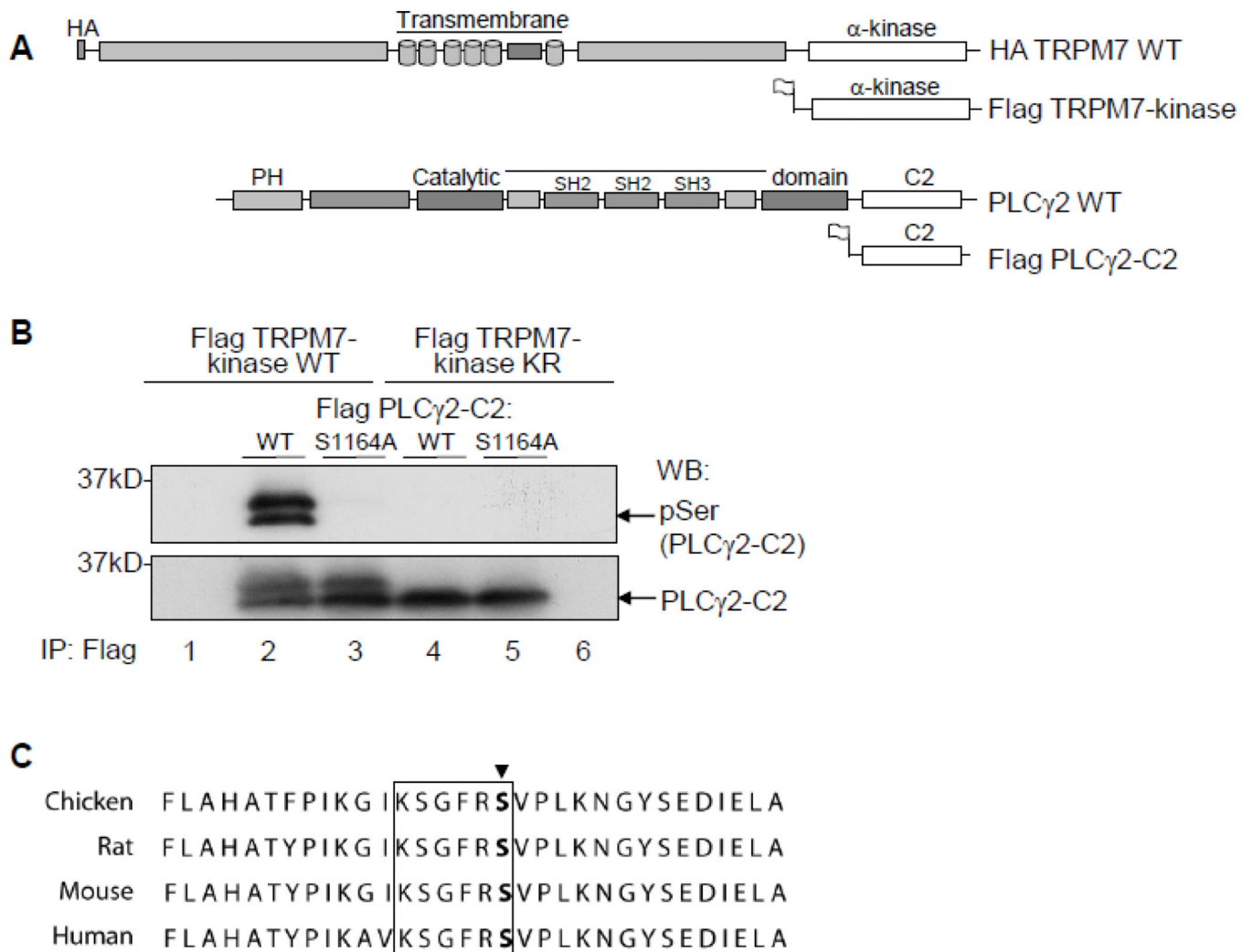


FIGURE 1. The PLC γ 2-C2 domain residue, Ser1164, is phosphorylated by TRPM7-kinase
A. Schematic of the recombinant TRPM7 and PLC γ 2 constructs used. **B.** Phosphorylation of PLC γ 2 determined using HEK293 cells transiently transfected with N-terminally Flag-tagged constructs of PLC γ 2-C2 WT, PLC γ 2-C2 S1164A, TRPM7-kinase WT or TRPM7-kinase dead (KR). Flag dual-immunoprecipitations performed followed by *in-vitro* phosphorylation reactions. Detection of Ser1164 phosphorylation in PLC γ 2 was determined after SDS-PAGE, and immunoblotting with anti-Phospho-Ser motif antibody. **C.** Alignment of a C-terminal portion of PLC γ 2 for chicken, mouse, rat, and human is shown, site Ser1164 marked by arrow, motif specific region is boxed in black.

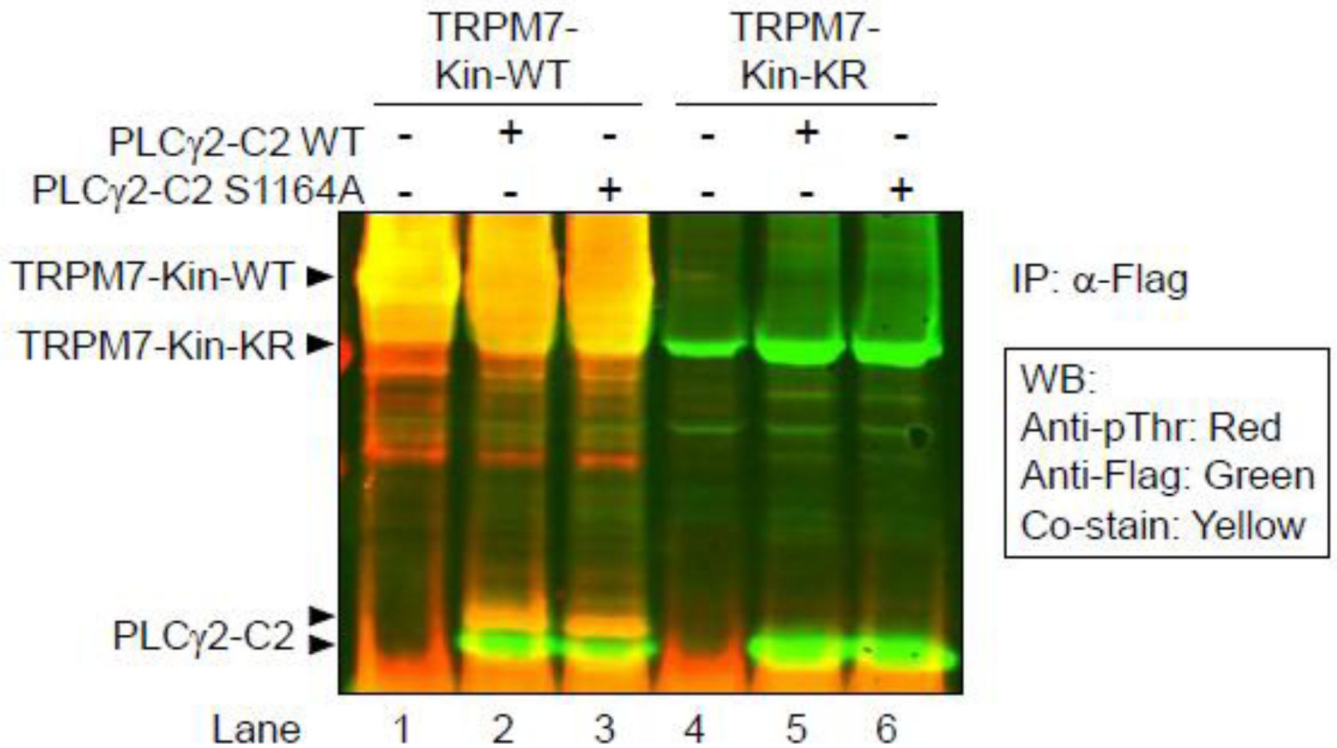


FIGURE 2. TRPM7-kinase not only phosphorylates Ser1164 in PLC γ 2-C2, but also a Thr residue(s)

Phosphorylation of PLC γ 2 determined using HEK293 cells transiently transfected with N-terminally Flag-tagged constructs of PLC γ 2-C2 WT, PLC γ 2-C2 S1164A, TRPM7-kinase WT or TRPM7-kinase dead (KR). Flag dual-immunoprecipitations performed followed by *in vitro* phosphorylation reactions. Detection of Ser1164 phosphorylation in PLC γ 2 was determined after SDS-PAGE, and immunoblotting with an anti-phospho-Thr antibody. Positive signals on the Western blot membrane were visualized using the Li-Cor Odyssey Infrared Imaging System.

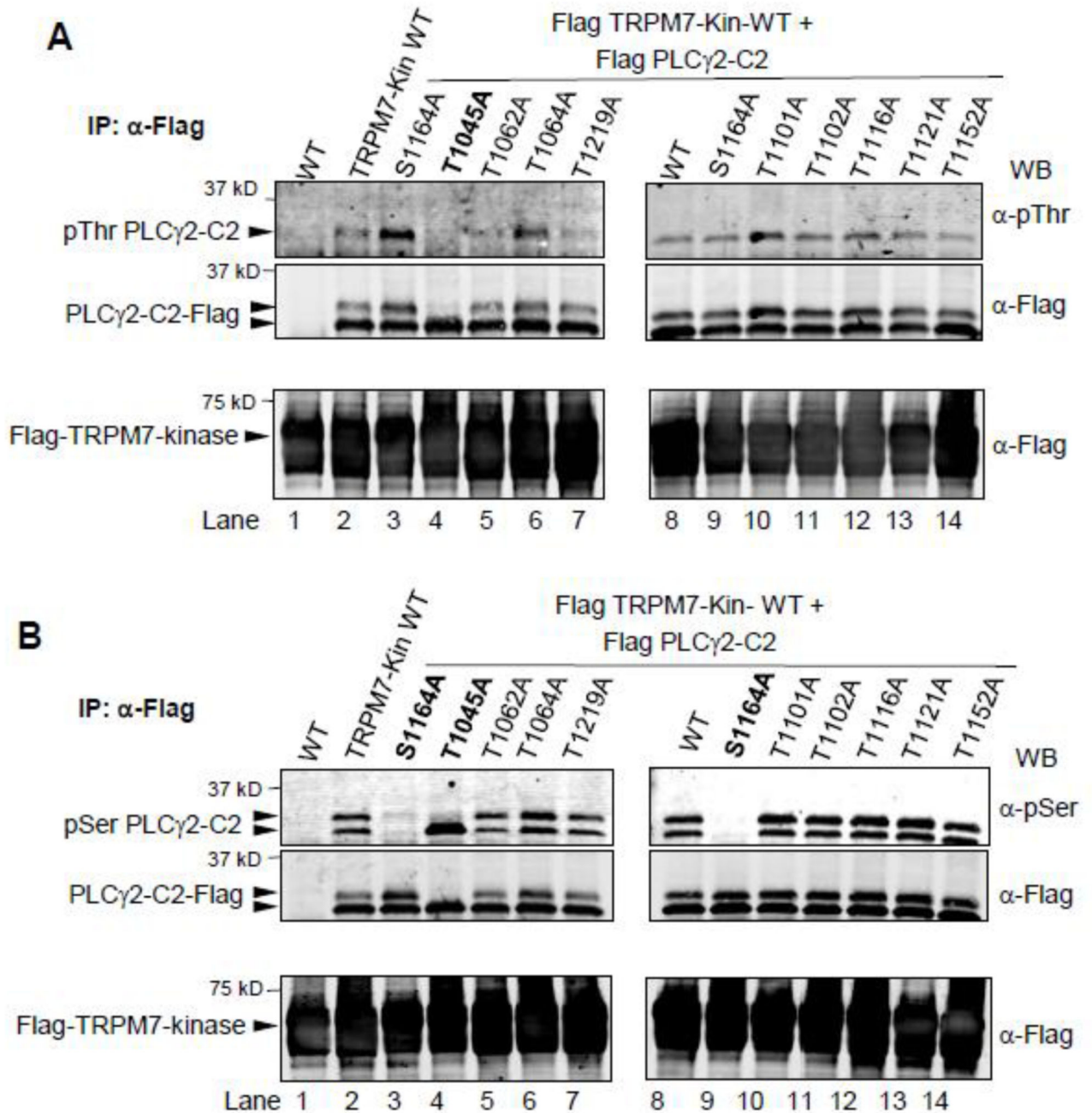


FIGURE 3. TRPM7-kinase phosphorylates Thr1045 in the linker region between the catalytic and C2-domain of PLC γ 2

A. Phosphorylation of PLC γ 2-C2 determined using HEK293 cells transiently transfected with N-terminally Flag-tagged constructs of PLC γ 2-C2 WT or indicated mutants, TRPM7-kinase WT or TRPM7-kinase dead (KR). Flag dual-immunoprecipitations performed followed by *in-vitro* phosphorylation reactions. Detection of threonine phosphorylation of PLC γ 2-C2 WT and mutants was determined after SDS electrophoresis, and immunoblotting

with antibody against phospho-Thr or Flag. *B*. Same experimental design as in *A*, except that immunoblotting was performed using antibody against anti-Phospho-Ser motif or Flag.

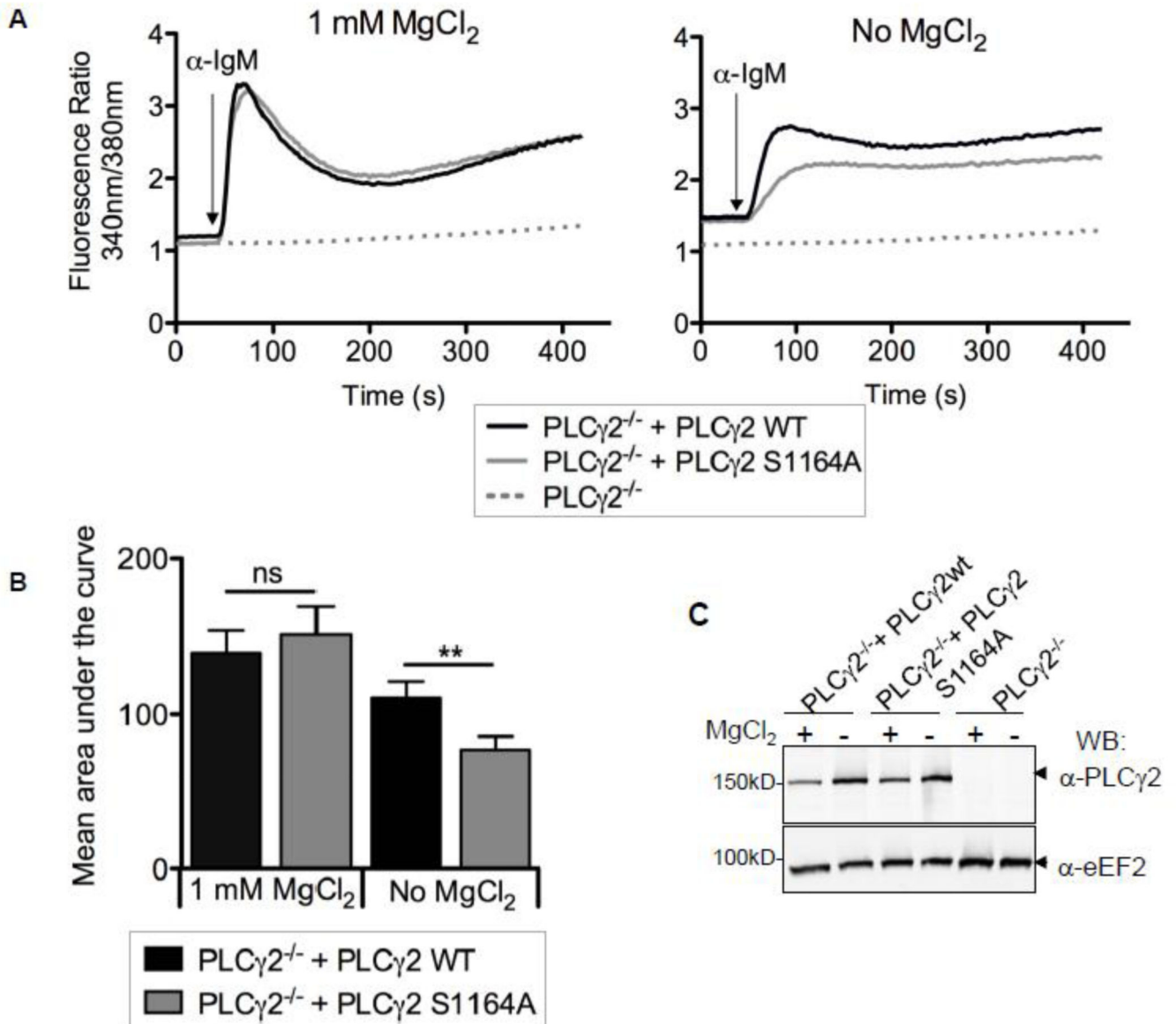


FIGURE 4. Under hypomagnesic conditions BCR mediated Ca²⁺-responses are reduced in PLCγ^{2-/-} DT40 cells complemented with PLCγ²-S1164A

A, Changes in free cytosolic Ca²⁺ in PLCγ^{2-/-} cells alone or with stable expression of PLCγ² WT or PLCγ² S1164A. Cell lines were cultured in 0 or 1 mM MgCl₂ for 15–25 h and loaded with Fura-2 for 30 min. Cells were stimulated with 1.2 μg anti-chicken IgM and analyzed by a fluorometer. Shown traces are representative of 4 separate experiments. **B**, Quantification of the results presented in **A** and **B** where bars represent the mean area under the curve and a paired two-tailed t-test was performed, mean ± SEM; ***p* < 0.01. **C**, Immunoblot of 2.5 × 10⁵ equivalent cells from whole cell lysates made from **A** and probed with anti-PLCγ² and anti-eEF2 (loading control).