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Cool-temperature-mediated activation of phospholipase C-[gamma]2 in the human hereditary disease PLAID

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Dear Professor Maurice,

thank you very much for the evaluation of and very positive response to our manuscript "Cool-temperature-mediated activation of phospholipase C- γ_2 in the human hereditary disease PLAID" by three expert reviewers of Cellular Signalling and for allowing us to submit a revised version of our work to the Journal. Thank you also very much for the extension of the deadline (hoping that you will allow me to use Canadian time and date for the submission).

In the following, we provide a point-by-point reply to the critical points raised by the reviewers:

(i) Reviewer 1: "The results section of the manuscript lacks subtitles. Addition of these would enhance the stringency of the presentation of the results and help the reader to digest the data more easily."

Authors' response: This was done in the revised manuscript.

(ii) Reviewer 1: "In all the figures where temperature shifts were analyzed, the authors choose the conventional way, where left starts with the lower temperature and right on the x axis are the higher temperature values. However, my personal opinion is that it would be more intuitive for the reader to put it the other way round. Left the physiologic temperature and right the reduced temperatures, which then lead to enhanced mutant signals. At least for me this would be a more intuitive way to show the data."

Authors' response: Although we clearly see and understand the point of the reviewer, we would very much like to adhere to the previous mode of presentation. This is mostly due to the fact that we feel that gradual changes in the temperature, such as those used in many of the figures (rather than the step changes used in the other figures) are clearer in the low to high temperature left to right presentation commonly used in physics. In particular, this format is commonly used for Q_{10} presentations such as the one in Fig. 3 in the field of temperature regulation of other proteins, e.g. of TRP channels.

(ii) Reviewer 1: "At least in the discussion the authors should address the question of temperature vs. ligand induced activation of Plcg2. The experiment with the constitutive Rac as activator is not entirely convincing. In vivo in mutant mice and in the patient the disease phenotype might be a mixture of temperature and ligand induce overreactivity, as the different mutants suggest." This point is related to Minor Point of **Reviewer 2:** "It would be interesting to know if upon stimulation (BCR triggering) at low temperature the highly active mutants can be further stimulated."

Authors' response: We have followed these important suggestions as much as we could possibly do in the COS-7 cell context, by analyzing the activation of exogenous wild-type versus $\Delta 20-22$ and $\Delta 19$ mutant PLC γ_2 by EGF receptors endogenously present in these cells. The results, which are shown in Fig. 11 of the revised manuscript, are intriguing because they imply that the mutant PLC γ_2 enzymes are resistant to stimulation by EGFR activation, both at 31 °C and 37 °C. This behavior is in striking contrast to that of wild-type PLC γ_2 with approx. 12-fold stimulation by EGFR activation at both 31 °C and 37 °C. Although activation of PLC γ_2 in COS-7 by activated EGFR differs from its activation in B lymphocytes by antigen-activated BCR or in other cells by other receptor tyrosine kinases, these results are similar to the loss of function effects seen in cells of PLAID patients and could provide hints to the mechanistic basis of the apparently disparate effects of the PLAID mutations, gain and loss of PLC γ_2 function.

(iii) Reviewer 1: "The octamer peptide PCI is fully conserved in PLC γ_1 and mediates cold sensitivity in PLC γ_2 . Which effect causes its deletion in PLC γ_1 ?"

Authors' response: We have not specifically addressed this question. However, since the PCI peptide is contained in the region deleted in the PLC γ_1 mutant " $\Delta 20-22$ ", we would predict that the functions of PLC $\gamma_1\Delta PCI$ would be similar to those of PLC $\gamma_1\Delta 20-22$ (cf. Fig. 4A), such as the functions of PLC $\gamma_2\Delta PCI$ resemble those of PLC $\gamma_2\Delta 20-22$ (cf. Fig. 4B, left). However, we do not feel that the octamer mediates cold sensitivity in PLC γ_2 , since it is maintained in PLC $\gamma_2\Delta 19$, which is nevertheless sensitive to activation by cooling (cf. Fig. 8B).

(iv) Reviewer 1: "The authors could provide data on the mobilization of Ca²⁺ upon the temperature shift. This would strengthen the data on IP₃ measurements."

Authors' response: We appreciate this suggestion of the reviewer. However, in other studies that we have performed on PLC γ_2 , we have always seen a close correlation between inositol phosphate formation and increase in cytosolic Ca²⁺, at least upon acute activation of the enzyme. In the chronic activation setting used in most of the experiments shown here, we would anticipate technical difficulties in observing reliable changes in [Ca²⁺]_i for the PLC γ_2 PLAID mutants.

(v) Reviewer 3: "Attempts were made to provide a structural rational for the activation mediated by cool temperatures. The main limitation here is that the work relies on indirect experiments that measure enzyme activity of PLC γ_2 variants in transfected cells without any further support from other approaches. Conceptual schemes are helpful but experimental limitations have to be pointed out. This should be emphasized in discussion."

Authors' response: This was explicitly done on p. 19 of the revised manuscript.

(vi) Reviewer 3: "Several observations deserve some more emphasis even if they can not be easily explained at present. These include: -The data suggesting that PLC γ_1 variants harboring deletions are not regulated by cool temperatures and that regions corresponding to deletions in PLAID have a bigger role in auto-inhibition in this enzyme. -The data related to Ali5 mutation and activation by cool temperatures. This mutation is not in the region affected by PLAID, actually not in the regulatory region at all. Some link to the PH domain has been suggested but this is mechanistically unclear. Unexpected behavior of Ali5 variant on its own and the distinct position of the mutation need to be pointed out. -The fact that many deletions in PLC γ_2 SH region are further activated by cool temperatures."

Authors' response: All three points have been addressed in the text of the revised manuscript on pp. 11, 13 (top paragraph), and 13 (bottom paragraph).

(vii) Reviewer 3: "It should be stated that assumption (or expectation) has been made that observations based on COS cell transfections will be relevant for endogenous expression levels in B-cells."

Authors' response: This was done in the revised manuscript on p. 20 in the context of discussing the resistance of the two deletion mutants to activated EGFR in COS-7 cells [cf. (ii)].

An additional point not requested by the reviewers was the examination of the requirement of the spPH domain *per se* for cool temperature regulation of PLC γ_2 . We felt that this experiment needed to be done and think that its results should be reported in this manuscript (now in Fig. 10B) to avoid misinterpretations of the role of spPH in mediating the enzyme's response to cooling. The results are intriguing: while spPH exerts a striking regulatory role within the PLAID PLC γ_2 mutant PLC $\gamma_2\Delta 19$, it is not required for the cool temperature response of further truncated mutants, such as the bipartite mutant made up of fragments X and Y. We would be happy, if you allowed us to show this data and an accordingly revised version of the model now shown in Fig. 10C-H.

In summary, we believe that we have carefully considered and taken care of most, if not all of the referees' critical points. We sincerely hope that these changes make our work acceptable for publication in Cellular Signalling. Needless to say, we are extremely grateful to all three reviewers for their expert opinions and invaluable and important advice on the manuscript.

Yours sincerely,

Peter Gierschik

Cool-temperature-mediated activation of phospholipase C- γ_2 in the human hereditary disease PLAID

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Abbreviations: PLC, inositol-phospholipid-specific phospholipase C; SH2, Src homology domain 2; SH3, Src homology domain 3; PH, pleckstrin homology domain; spPH, split PH domain; PCI, phospholipase C inhibitor peptide; Rac, Ras-related C3 botulinum toxin substrate; BCR, B cell receptor; SA, specific array; PLAID, PLC γ_2 -associated antibody deficiency and immune dysregulation; TRP, transient receptor potential; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; Co., control; aa, amino acid

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ABSTRACT

Deletions in the gene encoding signal-transducing inositol phospholipid-specific phospholipase C- γ_2 (PLC γ_2) are associated with the novel human hereditary disease PLAID (PLC γ_2 -associated antibody deficiency and immune dysregulation). PLAID is characterized by a rather puzzling concurrence of augmented and diminished functions of the immune system, such as cold urticaria triggered by only minimal decreases in temperature, autoimmunity, and immunodeficiency. Understanding of the functional effects of the genomic alterations at the level of the affected enzyme, PLC γ_2 , is currently lacking. PLC γ_2 is critically involved in coupling various cell surface receptors to regulation of important functions of immune cells such as mast cells, B cells, monocytes/macrophages, and neutrophils. PLC γ_2 is unique by carrying three Src (SH) and one split pleckstrin homology domain (spPH) between the two catalytic subdomains (spPHn-SH2n-SH2c-SH3-spPHc). Prevailing evidence suggests that activation of PLC γ_2 is primarily due to loss of SH-region-mediated autoinhibition and/or enhanced plasma membrane translocation. Here, we show that the two PLAID PLC γ_2 mutants lacking portions of the SH region are strongly (> 100-fold), rapidly, and reversibly activated by cooling by only a few degrees. We found that the mechanism(s) underlying PLC γ_2 PLAID mutant activation by cool temperatures is distinct from a mere loss of SH-region-mediated autoinhibition and dependent on both the integrity and the pliability of the spPH domain. The results suggest a new mechanism of PLC γ activation with unique thermodynamic features and assign a novel regulatory role to its spPH domain. Involvement of this mechanism in other human disease states associated with cooling such as exertional asthma and certain acute coronary events appears an intriguing possibility.

Keywords: Phospholipase C- γ_2 ; Inositol phospholipid; Rac2 GTPase; Split PH domain; Autoinhibition; Cold temperature sensitivity

1. Introduction

Inositol-phospholipid-specific phospholipases C (PLCs) catalyse the formation of inositol 1,4,5-trisphosphate and diacylglycerol, and, at the same time, decrease the local or general plasma membrane abundance of their substrate, phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) [1]. The latter three molecules are important mediators of cellular signaling. An enormous variety of cell surface receptors regulates important cellular functions utilizing PLCs, ranging from G-protein-coupled receptors over certain ion channels to many transmembrane non-enzymes and enzymes, e.g. receptor tyrosine kinases. The mammalian PLCs are divided into six subfamilies, β , γ , δ , ϵ , ζ , and η . Analyses of PLC crystal structures have revealed that the catalytic mechanism of PLCs is well conserved between all PLC family members. Their activation by cell surface receptors involves both translocation of the soluble PLC enzymes to the plasma membrane, i.e. the site of their phospholipid substrate(s), and removal of intramolecular autoinhibition [2-5]. However, certain findings suggest further, still unknown regulatory mechanisms of PLC isozyme activation [1].

The two members of the PLC γ subfamily, PLC γ_1 and PLC γ_2 , control functions represented in many, if not all cell types, such as cell growth and differentiation, as well as migratory responses, but are also involved in highly specialized tasks [6]. Examples of the latter are those regulated by PLC γ_2 in cells of the immune system. PLC γ_1 and PLC γ_2 are activated by receptor and nonreceptor tyrosine kinases; PLC γ_2 is also activated by Rac GTPases [7]. In B lymphocytes, this interaction amplifies B-cell-receptor-mediated Ca²⁺ signalling [8]. The structures of the two PLC γ isozymes are unique in that the two catalytic subdomains X and Y are separated by a modular assembly comprising a split PH domain (spPHn and spPHc), two SH2 (SH2n and SH2c), and one SH3 domain. The whole assembly (spPHn-SH2n-SH2c-SH3-spPHc) is also referred to as specific array (γ SA). Studies on isolated γ SA structures showed that the split PH domains of PLC γ_1 and PLC γ_2 do not alter their three-dimensional shapes upon insertion of the entire PLC γ_1 SH2n-SH2c-SH3 region between the two PLC γ_1 spPH halves and upon peptide ligand binding to the insertion [9] or, in case of spPH of PLC γ_2 , upon its interaction with activated Rac2 [10]. These findings suggested that the PLC γ split PH domain is a more rigid, conformationally stiff element of γ SA and that it mediates PLC γ_2 activation mainly by allowing Rac2 to translocate the enzyme to the plasma membrane. Recent evidence suggests that the SH2c domain is a major determinant of PLC γ_1 autoinhibition and that activation of the enzyme by tyrosine phosphorylation at a site immediately downstream of the domain (Y⁷⁸³) proceeds by competition of the phosphorylated peptide with a so far unidentified site on the catalytic XY TIM barrel for binding to SH2c [11,12].

Alterations of the primary structures of PLC γ_1 and PLC γ_2 are involved in disease, both in humans and in animal models. Thus, point mutations in the human *PLCG1* gene have been linked to secondary, radiation-associated angiosarcoma [13] and to cutaneous T cell lymphoma [14]. Two mouse models of autoimmunity and autoinflammation, designated Ali5 and Ali14, have been described, which are caused by gain-of-function point mutations of PLC γ_2 , D⁹⁹³G and Y⁴⁹⁵C, respectively [15-17]. The Ali5 mutation also gives rise to platelet hyperreactivity and a prothrombotic phenotype in mice [18]. Recently, deletion of exon 19 or exons 20-22 of the human *PLCG2* gene has been shown to cause a novel human hereditary disease characterized by cold urticaria, immunodeficiency, and autoimmunity, designated PLAID for PLC γ_2 -associated antibody deficiency and immune dysregulation [19]. In affected individuals, only very subtle skin cooling, such as the one caused by a single tear rolling down the cheek at room temperature, causes urticarial wheals and flares within one minute [20]. A related, but distinct human disease, predominantly characterized by autoinflammation and designated APLAID for autoinflammatory PLAID, is caused by a gain-of-function point mutation, S⁷⁰⁷Y, located in SH2c of PLC γ_2 ([21], cf. [22], for a more comprehensive review). Although some effects of decreasing temperature on functions downstream of PLC γ_2 have been documented in cells from PLAID patients [19], understanding of cool temperature regulation of the enzyme that is affected by the deletion mutations at first hand, PLC γ_2 , is currently lacking. Here, we show that the two PLC γ_2 mutants identified in PLAID patients, PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ (Fig. 1A), are exquisitely sensitive to cooling and that the magnitude of the response is unprecedented in that it goes far beyond those previously observed for many other signaling proteins sensitive to temperature changes, such as the transient receptor potential (TRP) cation channels [23]. The results suggest that PLAID PLC γ_2 mutants are activated by only minute decreases in temperature by a novel mechanism that is primarily mediated by the split PH domain and distinct from a loss of autoinhibition.

2. Material and methods

2.1. Material

The mouse monoclonal antibody 9B11 reactive against the c-Myc epitope (EQKLISEEDL) and the polyclonal antiserum reactive against human PLC γ_2 raised in rabbits (sc-407) were obtained from Cell Signaling Technology and Santa Cruz, respectively. The mouse monoclonal antibody AC-15 reactive against β -actin (A1978), human epidermal growth factor (E9644), and cycloheximide (C7698) were obtained from Sigma.

2.2. Construction of vectors

The construction of complementary DNAs encoding c-Myc-epitope-tagged human PLC γ_1 (1291 aa, accession number ABB84466), human PLC γ_2 (1265 aa, accession number NP_002652), and the spPH domain chimera PLC γ_2 -PH11 is described in [24]. The cDNAs of PLC $\gamma_2\Delta 19$ (deletion of exon 19, aa 646-685), PLC $\gamma_2\Delta 19$ -PH12, PLC $\gamma_2\Delta 19$ -PH21, PLC $\gamma_2\Delta 19$ -PH11 [with one or both portions of the PLC γ_2 spPH domain (aa 468-513 and aa 849-914, respectively) replaced in PLC $\gamma_2\Delta 19$ by the corresponding regions of PLC γ_1 (aa 482-527 and aa 872-937, respectively)], PLC $\gamma_1\Delta 19$ [deletion of PLC γ_1 residues corresponding to residues 646 to 685 of PLC γ_2 (aa 668-707)], PLC $\gamma_2\Delta SH$ (deletion of the SH2n-SH2c-SH3 region, aa 515-840), PLC $\gamma_2\Delta SH2c$ (deletion of the C-terminal SH2 domain, aa 639-766), and PLC $\gamma_2\Delta PCI$ (aa 726-733) were constructed by *in vitro* mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (200521, Agilent Technologies). The deletion of exons 20-22 in PLC γ_2 (PLC $\gamma_2\Delta 20$ -22, aa 686-806), of the PLC γ_1 residues corresponding to residues 686 to 806 of PLC γ_2 (aa 708-828; PLC $\gamma_1\Delta 20$ -22)], and of the PLC γ_2 specific array (PLC $\gamma_2\Delta SA$, aa 476-908) was performed using the PCR overlap extension method. The introduction of point mutations was performed by *in vitro* mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit according to the manufacturer's instructions. For the insertion of γ_2 SH domains into PLC $\gamma_2\Delta SH$, a linker containing an AvrII restriction site (GCCCTAGG, AvrII site underlined) was introduced into the deletion site of PLC $\gamma_2\Delta SH$. The SH domains (SH2n, aa 515-638, SH2n, aa 637-747, SH3, aa 767-839, SH2n-SH2c, aa 515-747, SH2c-SH3, aa 637-840) were amplified with primers containing an AvrII restriction site on either end and inserted into PLC $\gamma_2\Delta SH$ by restriction and ligation. The linker introduced five additional residues in positions 515-519 of the protein. There were no functional differences between the PLC $\gamma_2\Delta SH$ mutants with and without these residues. Complementary DNAs encoding PLC γ_2 -X^{PHn} (aa 1-514) and PLC γ_2 -Y^{PHc} (aa 841-1265) were amplified by PCR. Both fragments were c-Myc-tagged at their C-termini. DNAs encoding PLC γ_2 -X (aa 1-470) and PLC γ_2 -Y (aa 914-1265) were amplified by PCR. PLC γ_2 -X and PLC γ_2 -Y were c-Myc-tagged at their N- and C-termini, respectively. The primer sequences and PCR protocols are available from the authors upon request.

2.3. Cell culture and transfection

COS-7 cells were maintained at 37 °C in a humidified atmosphere of 90 % air and 10 % CO₂ in Dulbecco's modified Eagle's medium (41965-039, Gibco) supplemented with 10 % (v/v) fetal calf serum (10270-106, Gibco) and 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from PAA Laboratories, Cölbe, Germany). Prior to transfection, COS-7 cells were seeded into 24-well plates at a density of 0.75 x 10⁵ cells/well, and grown for 24 h in 0.5 ml of medium/well. For

transfection, plasmid DNA (500 ng/well) was diluted in 50 µl jetPRIME[®] buffer and 1 µl of jetPRIME[®] Reagent (114-15, Polyplus Transfection, Illkirch, France) was added according to the manufacturer's instructions. The total amount of DNA was maintained constant by adding empty vector. Four h after the addition of the DNA-jetPRIME[®] complexes to the dishes, the medium was replaced by fresh medium, and the cells were incubated for a further 20 h at 37 °C and 10 % CO₂.

2.4. Radiolabeling of inositol phospholipids and analysis of inositol phosphate formation

Twenty four hours after transfection, COS-7 cells were washed once with 0.3 ml/well of Dulbecco's PBS (PAA Laboratories) and then supplied with 0.2 ml/well of Dulbecco's modified Eagle's medium containing supplements as described under *Cell culture and transfection*, and additionally supplemented with 25 mM HEPES and 2 mM sodium pyruvate (both from PAA Laboratories) to maintain the pH of the medium [25], 2.5 µCi/ml *myo*-[2-³H]inositol (NET1156005MC, Perkin-Elmer), and 10 mM LiCl. The cells were incubated for 20 h in this medium in individual incubation chambers in ambient atmosphere at temperatures ranging from 25 °C to 39 °C, washed once with 0.2 ml/well of Dulbecco's PBS, and then lysed by addition of 0.2 ml/well of 10 mM ice-cold formic acid. After keeping the samples for 30 min at 4 °C, 0.3 ml/well of 10 mM NH₄OH was added for neutralization, and the sample was centrifuged for 5 min at 20,000 x g. The supernatants were loaded onto columns containing 0.5 ml of Dowex[®] 1 x 8-200 ion exchange resin (217425, Sigma) that had been converted to the formate form and equilibrated with H₂O. The columns were washed once with 3 ml of H₂O and twice with 3.5 ml each of 60 mM sodium formate and 5 mM sodium tetraborate. Inositol phosphates were eluted with 3 ml of 1 M ammonium formate and 100 mM formic acid. The eluate was supplemented with 15 ml of scintillation fluid (Quicksafe A, 1008000, Zinsser Analytic, Frankfurt, Germany) and the radioactivity was quantified by liquid scintillation counting.

2.5. Construction of individual incubation chambers

Chambers allowing the temperature-controlled incubation of individual 24-well tissue culture plates (92424; TPP, Switzerland) were custom assembled using 15 x 12 x 23 cm Styrofoam containers (Schaumaplast, Reilingen, Germany) equipped with circuits made up of one Velleman VM148 thermostat control module (190655-62) and one Dallas DS18S20-55 temperature sensor with digital output (176168-62) to control two serially connected heating foils (532878-62; all from Conrad Electronic, <http://www.conrad.de>) to be placed on either side of the tissue culture plate during incubation. To allow for additional external, analogous control of the temperature, a Ø 10 mm hole was drilled into the wall of the container and the tissue culture plate to allow insertion of a thermometer into one well. Power supply to up to 8 individual chambers was through a TDK-Lambda

LS75-12 AC/DC converter unit (511823-62; Conrad). A construction guidance including a circuit diagram is available from the authors on request.

2.6. Inositol phospholipid analysis

Inositol phospholipids were extracted from transfected cells and analyzed as before [17]. COS-7 cells were grown and radiolabeled in 6-well plates. At the end of the radiolabeling procedure, 10 µl of the medium supernatant were placed in a scintillation vial with 3 ml of Quicksafe A liquid scintillator (Zinsser Analytic) and the radioactivity was quantified by liquid scintillation counting. The radiolabeled cells were lysed by addition of 1.2 ml of 4.5 % (v/v) perchloric acid. After incubating the samples for 30 min on ice, they were scraped into 1.5 ml reaction tubes and centrifuged at 4 °C for 20 min at $3,700 \times g$. Supernatants and pellets were separated. The pellets were resuspended in 100 µl of water and 375 µl of chloroform/methanol/HCl (100:200:15) was added. The samples were vortexed, and an additional 125 µl of chloroform and 125 µl of 0.1 M HCl were added. After further vortexing, the samples were centrifuged at room temperature for 10 min at $700 \times g$. Fifty µl each of the lower, chloroformic phase containing the inositol phospholipids were subjected to liquid scintillation counting as described above.

2.7. Determination of the 10-degree temperature coefficients

According to Hille [26], the 10-degree temperature coefficient, Q_{10} , of a biological process can be calculated for an arbitrary temperature interval ΔT from

$$Q_{\Delta T} = (Q_{10})^{\Delta T/10}$$

Using $\Delta T = T_i - T_{ref}$ and $Q_{\Delta T} = \frac{A_i}{A_{ref}}$, where T_i and T_{ref} are the individual and a reference temperatures and A_i and A_{ref} are the individual and a reference activity, this equation can be rewritten to

$$\log_{10} \left(\frac{A_i}{A_{ref}} \right) = 0.1(T_i - T_{ref})\log_{10}(Q_{10})$$

$$\log_{10} \left(\frac{A_i}{A_{ref}} \right) = 0.1\log_{10}(Q_{10})T_i - 0.1\log_{10}(Q_{10})T_{ref}$$

Upon plotting the T_i vs. the $\log_{10} \left(\frac{A_i}{A_{ref}} \right)$, the Q_{10} value(s) can be calculated from the slopes of the linear portions of the resultant graphs.

2.8. Miscellaneous

Curve fitting was done using GraphPad Prism, version 4.03 (GraphPad Software, San Diego, CA, USA). In Fig. 3, the global curve fitting procedure was applied, where the extra sum of squares F-test is employed to determine whether the best-fit values of the two parameters differ between data sets. The simpler model was selected unless the P value was less than 0.05. The propagation of errors was calculated as outlined in [27]. The various PLC γ isoforms were tested in this study multiple times in many different combinations, including many that are not presented in this work. The experiments specifically shown herein were repeated two to three times throughout. Data from representative experiments are presented as means $\pm$ standard error of triplicate determinations. Control curves were done in Figs. 4B and 7 to confirm that results shown in different panels of the same figure are comparable. The intensities of immunoreactive bands on Western blots corresponding to PLC γ_2 isozymes carrying a c-Myc epitope were within the range allowing semiquantitative comparisons, as shown by control experiments using purified c-Myc-tagged wild-type PLC γ_2 . Samples to be analyzed by Western blotting were taken, *quasi* as a fourth replicate, from the same plate as and immediately adjacent to the samples taken in triplicate for functional analysis. Using this protocol and paying meticulous attention to experimental detail, we have not experienced variations in gel loading of these samples (*cf.* Fig. 5B).

3. Results

3.1. The PLC γ_2 deletion mutants PLC γ_2 D19 and PLC γ_2 D20-22 are specifically activated by cool temperatures

PLC γ_2 Δ 19 and PLC γ_2 Δ 20-22 were expressed in COS-7 cells at 37 °C and the cells were then radiolabeled with [3 H]inositol at temperatures ranging from 39 °C to 25 °C, followed by measurement of inositol phosphate formation. Fig. 1B shows that the two PLC γ_2 deletion mutants caused slight increases in inositol phosphate formation in comparison to the wild-type enzyme at 37 °C. Consistent with earlier results [19], these increases were approximately 2.8- and 3.6-fold for PLC γ_2 Δ 19 and PLC γ_2 Δ 20-22, respectively, relative to the increase over basal activity observed for wild-type PLC γ_2 . Much more strikingly, however, there was a marked stimulation of inositol phosphate formation when cells expressing either deletion mutant were incubated at only slightly lower temperatures. In both cases, there was a biphasic stimulatory response with declining temperatures, with a maximum at 31 °C and a gradual reduction upon further cooling to 25 °C. There was only a modest monophasic decrease in inositol phosphate formation in cells expressing wild-type PLC γ_2 and in control cells when the temperature was reduced from 39 °C to 25 °C. Strikingly, at 31 °C, the absolute increase in inositol phosphate formation in cells expressing PLC γ_2 Δ 19 and PLC γ_2 Δ 20-22 over basal activity of mock-

transfected control cells was enhanced approx. 480 ± 91 - and 430 ± 84 -fold (means $\pm$ SEM) relative to the increase over basal inositol phosphate formation observed in cells expressing wild-type PLC γ_2 . Fig. 1C shows that the expression of wild-type PLC γ_2 decreased with decreasing incubation temperature, while the expression of both PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ took slight increases at intermediate temperatures ranging from 35 °C to 31 °C and from 35 °C to 25 °C, respectively. While reduced expression of wild-type PLC γ_2 at lower temperatures may explain, at least in part, the monophasic decrease in inositol phosphate formation by this enzyme, the limited magnitude of the changes observed in Fig. 1C for PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ argues against a critical role of fluctuating enzyme expression in the marked changes of inositol phosphate formation enzyme activity evident in Fig. 1B.

3.2. The activation of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ by subphysiological temperatures occurs after protein synthesis and is reversible.

The influence of a decrease in incubation temperature on the abundance of inositol phospholipids in mock-transfected COS-7 cells and cells expressing either wild-type or $\Delta 19$ mutant PLC γ_2 is shown in Figs. 1D and E. PLC γ is capable of hydrolyzing PtdIns, PtdIns(4)*P*, PtdIns(4,5)*P*₂ [28]. There was no increase in the abundance of PLC γ substrate inositol phospholipids upon cooling. Instead, a monophasic loss was apparent in all three cases upon decreasing the temperature to 25 °C to approximately 13 % of the levels observed at 37 °C. This behavior was largely independent of whether or not wild-type PLC γ_2 or PLC $\gamma_2\Delta 19$ was expressed in the cells. While this loss may reflect slower synthesis of the substrate phospholipids at lower temperatures and may explain some of the decline in inositol phosphate formation by PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ at temperatures below 31 °C (*cf.* Fig. 1B), it argues against increased substrate availability causing the dramatic stimulation of the PLC γ_2 deletion mutants upon cooling from 37 °C to 31 °C (*cf.* Fig. 1B).

The next experiment was designed to examine whether stimulation of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ by cool temperatures occurs before or after synthesis of the mutant PLC γ_2 proteins. To this end, cells already containing either wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, or PLC $\gamma_2\Delta 20-22$ (as a result of a 24-h transfection period at 37 °C) were treated for a further 20 h (i.e. during the [³H]inositol radiolabeling phase) with or without cooling from 37 °C or 31 °C, both in the absence and in the presence of 100 µg/ml cycloheximide. Using this protocol, we expected that cycloheximide would block the stimulatory effect of cooling on inositol phospholipid hydrolysis by the mutants, if cooling were to be effective prior to or during recombinant protein synthesis. Fig. 2A shows that this was not the case. Thus, cooling to 31 °C caused marked increases in inositol phosphate formation, regardless of whether cycloheximide was absent or present during the second phase. Fig. 2B shows that, both at 37 °C and

at 31 °C, cycloheximide prevented the increase in abundance of wild-type and deletion mutant PLC γ_2 during the second phase (in comparison to the control samples obtained after the initial phase), indicating that cycloheximide was in fact effective as a protein biosynthesis inhibitor in this experiment. Taken together, these results indicate that the stimulatory effect of subphysiologic temperatures on deletion mutant PLC γ_2 activity occurs after protein synthesis.

To examine whether the stimulatory effects of the $\Delta 19$ and $\Delta 20-22$ deletions on PLC γ_2 activity were reversible, wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, and PLC $\gamma_2\Delta 20-22$ were incubated for the last four hours of the transfection protocol, i.e. in the absence of radiolabeled inositol phospholipid substrate, at either 37 °C or 31 °C and then radiolabeled with [^3H]inositol at one of the two temperatures. Fig. 2D shows that the three different incubation protocols had little, if any, effect on the expression of each of the three enzymes. As shown in Fig. 2C, preincubation of the three enzymes at 31 °C had no effect on their ability to promote inositol phosphate formation. Only when cells were incubated at 31 °C in the presence of [^3H]inositol, enhanced inositol phosphate formation was evident by the two deletion mutants, in contrast to wild-type PLC γ_2 . A time-course of the changes of inositol phosphate formation by wild-type and mutant PLC γ_2 upon changes in incubation temperature from 37 °C to 31 °C and *vice versa* is shown in Figs. 2E and 2F. Both deletion mutants were activated with no apparent lag time with cooling to 31 °C. However, deactivation of both enzymes by warming to 37 °C required a lag time of approximately 10 min to come into effect. Thus, activation of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ by cool temperatures is a rapid and slowly, but fully reversible process.

The determination of the 10 °C temperature coefficient (Q_{10}) value, widely used to characterize the regulation of TRP channels by temperature [23], for wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, and PLC $\gamma_2\Delta 20-22$ is shown in Fig. 3. While only a single linear component with a Q_{10} value of 4.6 was evident for wild-type PLC γ_2 between 39 °C and 25 °C, both PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ displayed two separate phases of opposite signs and markedly distinct Q_{10} values. Specifically, while Q_{10} was as high as 6745 for the marked increase in activity between 39 °C and 33 °C, it was lower by more than three orders of magnitude and not different, by sign and by magnitude, from the Q_{10} value describing the decrease in activity of the wild-type enzyme, 4.6, between 31 °C and 25 °C. Thus, it appears that the major functional consequence of the two deletions is their increased activity upon cooling from 37 °C to approximately 31 °C. The decrease in activity observed at temperatures below 31 °C appears to be a property inherently present in the remaining elements of wild-type PLC γ_2 . The Q_{10} values of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ exceed those reported for other temperature-regulated proteins, such as thermoTRPs (with values ranging from 10 to > 100 [23]). However, higher Q_{10} values are not without precedence. For example, the step of heat damage to the development of the posterior crossvein of *Drosophila* that is most sensitive to increasing temperature showed a Q_{10} of about 360 [29].

3.3. Cool-temperature-mediated activation of PLC γ_2 D19 and PLC γ_2 D20-22 is distinct from loss of SH-region-mediated autoinhibition.

The structural organization of PLC γ_2 between its two catalytic subdomains X and Y is very similar to that of its close relative PLC γ_1 [6]. To address the question, whether deletion in PLC γ_1 of residues corresponding to those encoded by exons 19 and 20-22 of *PLCG2* has similar functional consequences, the relevant mutants of human PLC γ_1 were produced. Since the human *PLCG1* and *PLCG2* genes exhibit distinct patterns of genomic organization, the deletions in PLC γ_1 do not correspond to specific exons in *PLCG1*. Hence, the mutants are designated PLC γ_1 " Δ 19" and PLC γ_1 " Δ 20-22". Fig. 4A shows that both PLC γ_1 " Δ 19" and PLC γ_1 " Δ 20-22", in contrast to their PLC γ_2 counterparts, were already highly constitutively active at 37 °C (54- and 37-fold increases in inositol phosphate formation, respectively, in comparison to the increase caused by wild-type PLC γ_1 relative to control). Furthermore, only minor changes in their activity were observed upon reduction of the incubation temperature from 37 °C to 27 °C. There were only minor changes in inositol phosphate formation by wild-type PLC γ_1 . Fig. S1A shows that there were minor, if any changes in protein expression of the PLC γ enzymes with decreasing temperatures, except for PLC γ_1 " Δ 20-22" and PLC γ_2 Δ 20-22, which showed increased expression. It seems possible on the basis of these findings that the regions of PLC γ_1 corresponding to the regions deleted in PLC γ_2 in PLAID exert a stronger autoinhibitory influence on PLC γ_1 than their PLC γ_2 counterparts on PLC γ_2 and that this difference blunts the stimulatory response of PLC γ_1 " Δ 19" and PLC γ_1 " Δ 20-22" to cooling. This possibility notwithstanding, the results indicate that the temperature sensitivities of PLC γ_1 and PLC γ_2 carrying deletions corresponding to exons 19 and 20-22 of *PLCG2* are distinct.

The region encoded by exons 20 to 22 of *PLCG2* contains an octapeptide (YRKMRLRY) at the end of SH2c that is absolutely conserved in PLC γ_1 and has previously been shown to mediate inhibition of PLC γ_2 *in trans* and *in cis* [30,31]. To determine whether deletion of the octamer, designated PCI (for phospholipase C inhibitor), is sufficient to mediate sensitivity of PLC γ_2 to cool temperatures, the deletion mutant PLC γ_2 Δ PCI was functionally compared to PLC γ_2 Δ 20-22 and wild-type PLC γ_2 (Fig. 4B, *left panel*). In addition, three other mutants carrying deletions known to promote constitutive activity of PLC γ_2 at 37 °C, PLC γ_2 Δ SA, PLC γ_2 Δ SH, and PLC γ_2 Δ SH2c, were characterized (Fig. 4C, *right panel*). PLC γ_2 Δ PCI was analyzed again in the latter experiment as a control to ensure that the results shown in the two panels of Fig. 4B are comparable. Figs. 4B and S1B show that PLC γ_2 Δ PCI shared most features of its temperature sensitivity with PLC γ_2 Δ 20-22 and, by extension, with PLC γ_2 Δ 19 (*cf.* Fig. 1B): only a limited enhancement of its activity at 37 °C, a marked activation upon lowering the incubation temperature from 37 °C to 31 °C, and a decrease in activity at

temperatures below 31 °C. In contrast, PLC $\gamma_2\Delta$ SA, PLC $\gamma_2\Delta$ SH, and PLC $\gamma_2\Delta$ SH2c, although exhibiting constitutive activity at 37 °C, showed an only modest, further increase in activity with cooling, which was monophasic for PLC $\gamma_2\Delta$ SH2c and only vaguely biphasic for PLC $\gamma_2\Delta$ SA and PLC $\gamma_2\Delta$ SH.

At first sight, the constitutive activities of the mutants PLC $\gamma_2\Delta$ SA, PLC $\gamma_2\Delta$ SH, and PLC $\gamma_2\Delta$ SH2c observed at 37 °C in Fig. 4B, *right panel*, appeared rather limited in comparison to the constitutive activities reported earlier for similar mutants of PLC γ_1 and PLC γ_2 [31,32]. Therefore, the activities at 37 °C of wild-type PLC γ_2 , PLC $\gamma_2\Delta$ 19, PLC $\gamma_2\Delta$ 20-22, PLC $\gamma_2\Delta$ PCI, PLC $\gamma_2\Delta$ SH2c, PLC $\gamma_2\Delta$ SH, and PLC $\gamma_2\Delta$ SA were compared as a function of their expression levels in a more comprehensive analysis (Figs. 5A and B). The results show that, taking the relative abundance of the PLC γ_2 variants into account (Fig. 5B), PLC $\gamma_2\Delta$ PCI, PLC $\gamma_2\Delta$ SA, PLC $\gamma_2\Delta$ SH2c, and PLC $\gamma_2\Delta$ SH exhibited higher constitutive activity at 37 °C than PLC $\gamma_2\Delta$ 19 and PLC $\gamma_2\Delta$ 20-22.

The fact that either of the two PLC γ_2 mutants, PLC $\gamma_2\Delta$ 19 and PLC $\gamma_2\Delta$ 20-22, were activated by cool temperatures to a similar degree raised the question as to the effect of a combined deletion of the residues encoded by exons 19 through 22. Fig. 6 shows that, at 37 °C, the compound deletion mutant PLC $\gamma_2\Delta$ 19-22 displayed a markedly enhanced activity in comparison to either PLC $\gamma_2\Delta$ 19 or PLC $\gamma_2\Delta$ 20-22 alone, showing only marginal enhancements of their basal activities. The stimulatory effect of cooling to 31 °C was assayed at two expression levels of the deletion mutants. At both expression levels, the activity of PLC $\gamma_2\Delta$ 19-22 at 31 °C was much higher than the sum of the activities of PLC $\gamma_2\Delta$ 19 and PLC $\gamma_2\Delta$ 20-22. Hence, at both temperatures, 37 °C and 31 °C, and at the expression levels tested in this experiment, the deletions of *PLCG2* exons 19 and 20-22 synergized to promote activation of PLC γ_2 .

3.4. The mouse PLC γ_2 mutant Ali5 is also sensitive to activation by cool temperatures.

The symptoms observed in the mouse Ali5 and Ali14 disease models bear resemblance to the PLAID phenotype in some, but not in all respects [15,16,19,20]. In transfected COS-7 cells, the corresponding PLC γ_2 mutants, PLC γ_2^{Ali5} and PLC γ_2^{Ali14} displayed only slightly enhanced basal activity at 37 °C, whereas the compound mutant, PLC $\gamma_2^{\text{Ali5/Ali14}}$ clearly showed constitutively enhanced activity [17]. In Ali5 mice, autoimmune dermatitis commenced in the superficial layers of the paws and ears, i.e. in cool body regions [15]. This distribution resembles those of cutaneous granulomatous lesions in PLAID patients [19]. We therefore compared the effect of cool temperatures on the activity of the mutants PLC γ_2^{Ali5} , PLC γ_2^{Ali14} , and the compound mutant PLC $\gamma_2^{\text{Ali5/Ali14}}$. Figs. 7, *left panel*, and S2A show that all three mutants exhibited moderately enhanced activity at 37 °C (PLC $\gamma_2^{\text{Ali5/Ali14}}$ >

PLC $\gamma_2^{\text{Ali5}} > \text{PLC}\gamma_2^{\text{Ali14}}$) as described before [17]. Importantly, however, the three mutants displayed considerable differences in their sensitivities to cool temperatures, both in quantitative and in qualitative terms. Thus, while the response of PLC $\gamma_2^{\text{Ali5/Ali14}}$ to decreasing temperatures closely resembled the pattern observed for PLC $\gamma_2\Delta 20-22$ (Figs. 7, *center panel*, and S2B), PLC γ_2^{Ali14} showed only a minor, monophasic increase in activity, which was opposite to the decrease observed for wild-type PLC γ_2 (Figs. 7, *right panel*, and S2C). PLC γ_2^{Ali5} displayed an intermediate phenotype, nonetheless showing an almost 7-fold activation upon cooling from 37 °C to 31 °C (Fig. 7, *right panel*). These results are surprising since the *Plcg2* mutations underlying the Ali5 and Ali14 phenotypes cause point mutations at positions outside of the SH2n-SH2c-SH3 region. Perhaps, similar structural and, hence, functional alterations are brought about by distinct mutations of the enzyme. Nevertheless, the results suggest that some of the lesions noticed in the affected animals, such as inflammation in superficial skin layers, may in fact be caused by further activation of PLC γ_2^{Ali5} by cool temperatures. An intriguing question to be clarified by future investigation is whether PLC γ_2 activated by other means, e.g. by other mutations or by tyrosine phosphorylation, are also sensitive to cold temperatures.

A more comprehensive comparison of the effects of the various constituents of the SH2n-SH2c-SH3-spPHc region, alone or in combination, on PLC γ_2 constitutive activity and sensitivity to cool temperatures is shown in Fig. 8A. Unlike observed previously [31,32], deletion of SH3 also caused an increase in basal activity, which was even slightly higher (approximately 4.1-fold) than that monitored for deletion of SH2c (2.6-fold), but lower than that for deletion of all three SH domains (approximately 7.5-fold). The increases for the variants lacking two SH domains, PLC $\gamma_2\Delta\text{SH2nSH2c}$, PLC $\gamma_2\Delta\text{SH2cSH3}$, and PLC $\gamma_2\Delta\text{SH2nSH3}$, were approximately 3.6-, 1.9-, and 2.4-fold, respectively. While deletion of SH2n did not change the temperature sensitivity of PLC γ_2 relative to the wild-type enzyme and deletion of all three SH domains resulted in a PLC γ_2 mutant largely insensitive to cool temperature, all other deletion mutants were clearly sensitive. Maximal sensitivity (approximately 6.1-fold) was observed for PLC $\gamma_2\Delta\text{SH2cSH3}$, followed, in that order, by $\Delta\text{SH2nSH3}$, $\Delta\text{SH2nSH2c}$, ΔSH2c , and ΔSH3 . Note that the degrees of stimulation by cool temperatures was by far lower for these mutants than the degrees observed for the PLAID PLC γ_2 mutants $\Delta 19$ and $\Delta 20-22$ (*cf.* Fig. 1). Fig. 8B shows a schematic representation of the deletions within the SH2n-SH2c-SH3 region (aa 515-840).

3.5. *Cool-temperature-triggered activation of PLC γ_2 deletion mutants is controlled by, but does not necessarily require the split PH domain.*

PLC γ_2 is activated by tyrosine phosphorylation and by activated Rac GTPases. The experiment shown in Fig. 9A was designed to determine the effect of decreasing temperatures on the ability of constitutively active Rac2^{G12V} to activate PLC $\gamma_2\Delta 19$ in comparison to wild-type PLC γ_2 . At 37 °C, Rac2^{G12V} caused similar enhancements of the activity of both enzymes (Figs. 9A, *left panel*, and S3A). Lowering the incubation temperature, however, took a very different effect on Rac2^{G12V}-mediated activation of the two enzymes. While only minor changes were observed for wild-type PLC γ_2 , there was a progressive loss of the stimulatory effect of Rac2^{G12V} on PLC $\gamma_2\Delta 19$. This loss is unlikely to be due to exhaustion of the inositol phospholipids substrate at lower temperatures, since PLC $\gamma_2\Delta 19$ is well capable of producing even higher levels of inositol phosphates when expressed at higher density (Fig. 9A, *right panel*, and S3B). Likewise, it was unlikely that Rac2^{G12V} became limiting at low temperatures, since stimulation of wild-type PLC γ_2 was well retained even at the lowest temperature, 27 °C.

The activation of PLC γ_2 by Rac is mediated by the internal, split PH domain of the enzyme [10,24]. The interdependence of the stimulatory effects of Rac2^{G12V} and cooling evident in Figs. 9A and S3 prompted us to determine the role of this domain in PLC γ_2 deletion mutant activation by cooling. Alanine replacement in spPH of PLC γ_2 of W⁸⁹⁹, which is conserved in all PH domains [33] has previously been shown to result in a loss of PLC γ_2 stimulation by Rac, but not by tyrosine phosphorylation [31]. Figs. 9B and S4A show that the W⁸⁹⁹A replacement completely abrogated the response of the enzyme to cool temperature activation. Furthermore, replacement of one or the other half of the split PH domain of PLC γ_2 by the corresponding portions of PLC γ_1 , previously shown to block activation of the mutant enzymes by Rac, but to take no effect on their catalytic activity [24], also eliminated enzyme activation by cool temperatures. In contrast, replacement of both PH domains halves, which does not convey Rac sensitivity to PLC γ_2 [24], fully rescued the response of PLC γ_2 to cooling. These findings indicate that there is important structural interaction between the SH2n-SH2c-SH3 region and the surrounding spPH sequence. Two residues of the N-terminal half of the PLC γ_1 spPH domain, Y509 and F510 (*cf.* Fig. 9C), have been shown to be important for the ability of spPHn to cooperate with SH2c to maintain PLC γ_1 in an inactive conformation [17,34]. To examine the role of this site in the cool temperature response of PLC γ_2 , the two residues were substituted by alanine residues in PLC $\gamma_2\Delta 19$ carrying the entire spPH of PLC γ_1 (PLC $\gamma_2\Delta 19$ -PH11), thus generating the mutant PLC $\gamma_2\Delta 19$ -PH11-Y⁵⁰⁹A/F⁵¹⁰A. Consistent with earlier findings [17,34], there was a slight increase in basal activity of the latter mutant in comparison to PLC $\gamma_2\Delta 19$ -PH11. Remarkably,

however, and in striking contrast to PLC $\gamma_2\Delta 19$ -PH11, there was no response of the mutant to cooling. Y⁵⁰⁹ and F⁵¹⁰ of PLC γ_1 corresponds to Y⁴⁹⁵ and C⁴⁹⁶ of PLC γ_2 . Most interestingly, alanine replacement of either one of the latter residues within the PLC $\gamma_2\Delta 19$ (carrying its own, authentic spPH domain), caused a distinct loss of the enzyme's response to cooling, replacement of both Y⁴⁹⁵ and C⁴⁹⁶ led to an almost complete loss of this response (Figs. 9D and S4B). These results indicate that a functional split PH domain, not necessarily the one genuinely contained in PLC γ_2 , is required with the context of the PLAID PLC γ_2 deletion mutants to mediate their activation by cool temperatures.

The observation that PLC $\gamma_2\Delta$ SH, a mutant lacking the entire SH2n-SH2c-SH3 region, displays enhanced basal activity, but is largely insensitive to activation by cold is remarkable, because this mutant lacks all constituents thought to mediate autoinhibition of the enzyme (Fig. 8B). On the one hand, this finding clearly suggests that the activation pattern of the two PLAID mutants, PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20$ -22, is distinct from a mere loss of autoinhibition, and indicates, by extension, that it is based on a specific molecular mechanism(s). On the other hand, the observation is rather puzzling, because cold activation appears to be, at the same time, dependent on and independent of specific constituents of the SH2n-SH2c-SH3 region. However, given the importance of the split PH domain for cold activation of PLC γ_2 emerging from the experiments shown in Fig. 9B and D, the possibility remained that activation by cold temperatures is sterically and/or conformationally restricted in the mutant PLC $\gamma_2\Delta$ SH by the (artificial) covalent linkage between the two halves of the split PH domain (*cf.* Fig. 9C). To examine this possibility, we took advantage of earlier findings suggesting that PLC isozymes can be expressed as two independent polypeptide fragments containing either of the two catalytic subdomains, X and Y [35,36]. Thus, PLC $\gamma_2\Delta$ SH was expressed either as a single polypeptide and as two separate chains, encompassing either of the two catalytic subdomains X^{PHn} and Y^{PHc}, either one containing the corresponding half of the split PH domain, followed by functional analysis at 37 °C and 31 °C. Figs. 10A and S5 show that expression of the N-terminal or the C-terminal half of PLC γ_2 did not lead to enhanced inositol phosphate formation. In marked contrast, when both halves, X^{PHn} and Y^{PHc}, were coexpressed, basal inositol phosphate formation at 37 °C was markedly enhanced. More importantly, however, decreasing the incubation temperature to 31 °C caused a substantial enhancement of PLC activity, in striking contrast to cells expressing the Δ SH deletion mutant of PLC γ_2 , where no change in activity was observed. These results strongly suggest that the structural element(s) mediating cold activation of PLAID PLC γ_2 mutants reside outside the SH2n-SH2c-SH3 region and that its responsiveness to cool temperatures is blocked by covalent linkage between the two portions of the PLC γ_2 split PH domain.

To examine the requirement of the split PH domain in itself for cool-temperature-mediated activation of coexpressed X^{PHn} and Y^{PHc}, the coding regions of the split PH domain halves were

removed from the two fragments, resulting in fragments X and Y. The two fragments were expressed alone or together and functionally analyzed at 31 °C or 37 °C. Most intriguingly, in contrast to cells expressing either X or Y alone, cells coexpressing fragments X and Y displayed a marked enhancement of inositol phosphate formation by lowering the incubation temperature to 31 °C (Fig. 10B). This enhancement was similar in extent as the one observed for PLC $\gamma_2\Delta 20-22$ (approximately 32-fold *versus* 41-fold). Thus, although the split PH domain appears to control the cool temperature sensitivity of the PLAID PLC γ_2 mutants, it is not required *per se* for cool- temperature-mediated activation of the enzyme's core constituents. The expression of wild-type and $\Delta 20-22$ mutant PLC γ_2 as well as fragments X and Y was similar at 31 °C and 37 °C, respectively (Fig. S6).

3.5. The PLAID PLC γ_2 mutants are resistant to activation by EGF receptors endogenously expressed in COS-7 cells.

The rather puzzling concurrence of gain and apparent loss of function of PLC γ_2 in certain PLAID patient cells [19,37] prompted us to determine the effects of the two PLAID PLC γ_2 mutations on the response of the mutant enzymes to tyrosine kinase receptor activation. To this end, we made use of EGF receptor tyrosine kinases endogenously expressed in COS-7 cells and capable of both regulating several endogenous signalling functions and activating exogenous PLC γ_2 [38,39,17] and examined their ability to stimulate PLC γ_2 -mediated inositol phosphate formation. Fig. 11 shows that addition of 100 ng/ml of EGF caused similar (approximately 12-fold) increases in wild-type PLC γ_2 activity at 37 °C and 31 °C. In contrast, neither PLC $\gamma_2\Delta 20-22$ nor PLC $\gamma_2\Delta 19$ were affected by addition of EGF, regardless of whether the radiolabeling of cells and their treatment with EGF was done at 37 °C or 31 °C. The inability of EGF to mediate PLC γ_2 PLAID mutant stimulation at 37 °C was not due to lack of available enzyme substrate, since the two mutants were markedly sensitive to activation by cooling to 31 °C. Additional experiments (not shown) revealed that substrate depletion did also not occur in the presence of the mutant PLC γ_2 enzymes at 31 °C. Fig. S7 shows that the functional changes depicted in Fig. 11 were not due to changes of wild-type or mutant PLC γ_2 expression.

4. Discussion

The results presented in this work show that deletion of residues encoded by exons 19 and 20-22 from PLC γ_2 causes both a mild constitutive activation of inositol phosphate formation at 37 °C and a marked, several-hundred-fold enhancement of this activity in response to very small decreases in temperature upon expression of the mutant enzymes in intact cells. These functional responses are qualitatively and quantitatively similar, if not identical for both types of mutants. While the former changes are consistent with those reported earlier for transfected cells, the latter are much higher than

the small increases in $[Ca^{2+}]_i$ observed when peripheral blood B cells of PLAID patients are exposed to cold. In the latter case, differences to normal B cells were only observed at temperatures below 29 °C, not exceeding a maximum of an about two-fold increase at 21 °C [19]. The results shown here suggest that the primary defect of the mutant enzymes, i.e. sensitivity to stimulation by cool temperatures, emerges at temperatures only a few degrees below the normal body temperature. These findings are in agreement with clinical findings on patients with a deletion of exons 20-22, where only very subtle cooling, such as the one caused by a single tear rolling down the cheek of an affected family member at room temperature caused symptoms within one minute [20]. In additional experiments (not shown), we found that application of single drops of water (60 µl) to human skin caused a transient, evaporative-heat-loss-mediated decrease in skin temperature by about 6 °C. Of note, even at an ambient, comfortable temperature of 27 °C, the temperature in many cutaneous and subcutaneous regions is already somewhat lower than the esophageal core temperature of 37 °C [40], such that only minimal further temperature decreases by evaporative heat loss may suffice to cause maximal PLC γ_2 activation. The considerable decrease of the stimulatory effect observed at temperatures below 31 °C (Fig. 3) may explain, together with the decrease in inositol phospholipid synthesis (Fig. 1D) - why PLAID patients are typically negative in the cold stimulation time test (CSTT), involving cooling with ice, and upon cold-water immersion [19,20]. Under these conditions, the cooled tissues may reside at the enzyme-activating temperatures only very transiently, i.e. too shortly for allowing effective activation mutant PLC γ_2 . The time course of PLC γ_2 deletion mutant activation and its reversible nature closely matches the clinical observation on PLAID patients of an immediate onset of inflammatory symptoms upon evaporative skin cooling and a somewhat slower resolution of the symptoms developing over the course of 30 minutes of rewarming at room temperature [20]. Mouse PLC γ_2 carrying a gain-of-function mutation, D993G, in the catalytic region, PLC γ_2^{Ali5} , and causing spontaneous autoinflammation and autoimmunity shows a similar, albeit less dramatic response to cold temperatures. This suggests that some of the lesions noticed in the affected animals, such as inflammation in superficial skin layers, may in fact be caused by further activation of PLC γ_2^{Ali5} by cool temperatures. An intriguing question to be clarified by future investigation is whether PLC γ_2 activated by other means, e.g. by other mutations [21] or by tyrosine phosphorylation, are also sensitive to cold temperatures.

Three mechanisms, not necessarily mutually exclusive, should be considered to explain the sensitivity of the PLC γ_2 mutants to cold temperatures in intact cells. First, other cold-sensitive molecules endogenously present in COS-7 cells - and, possibly, in mast cells - might indirectly cause activation of the mutants, e.g. *via* a soluble mediator or *via* altered protein-protein-interaction patterns. Second, the mutants may be specifically enabled to sense temperature-mediated changes in the physical properties of the plasma membrane phospholipid bilayer containing the enzyme's

substrate(s). The third possibility is that an intrinsic conformational change is induced in mutant PLC γ_2 by temperatures between 37 °C and 31 °C. With regard to the first possibility, we would like to point out that, among the known cold-temperature-sensitive molecules, TRPA1, TRPM8, and TRPC5, the former two responded to cold temperatures only within lower temperature ranges [23] and endogenous expression of the latter two was not evident in COS-7 cells [39-44]. While we cannot at present formally exclude the second mechanism, we note that the phase transition temperature (T_m) of dipalmitoylphosphatidyl-choline (the most representative lipid in model membrane studies) in artificial phospholipid membrane vesicles is 41 °C [45] and thus outside the range of temperatures mediating activation of the PLC γ_2 mutants. However, the behavior of native plasma membranes may be different. More experimentation is required to elucidate the exact nature of the interplay among the mechanisms potentially involved in cool-temperature-mediated activation of PLAID PLC γ_2 mutants under conditions of physiological PtdIns(4,5) P_2 presentation as a substrate to PLC γ_2 in native plasma membranes of intact cells.

If the structural element mediating, directly or indirectly, the sensitivity of PLC γ_2 deletion mutants to cool temperatures in intact cells resides on the enzyme itself, the simplest interpretation of the results would be that removal of the portions encoded by exons 19 or 20-22 causes a loss of an autoinhibition, since the two regions overlap with regions identified before [11,12,31,32] or in this study (*cf.* Fig. 8) to be autoinhibitory at 37 °C. However, the results shown in Fig. 8 suggest that constitutive activity at 37 °C and stimulation by cool temperatures are not necessarily correlated with each other. While this would not formally exclude a loss of autoinhibition, acquisition of an autostimulatory mechanism sensitive to decreasing temperatures appears as an alternative explanation. At first glance, a structural element residing in the SH domain region of PLC γ_2 would appear a likely candidate for triggering such a stimulation. However, the deletion experiments shown in Fig. 8A revealed that none of the constituents of the entire SH2n-SH2c-SH3 region is specifically required for enzyme activation by cool temperatures and that even a deletion mutant lacking both of the short partial repeats shared between the two SH2 domains (PLC $\gamma_2\Delta$ SH2nSH2c) is activated by incubation at 31 °C.

Thus, the possibility remains that cool-temperature-sensitivity in intact cells resides either in the split PH domain or in the remainder of the enzyme, and that this sensitivity is specifically kept in check by either the intact, native SH region, by its SH2c-SH3 portion, or by a covalent linkage between the two split PH domain halves. At first sight, the fact that the cool-temperature-sensitivity is lost when non-homonymous split PH domain halves are present in PLC γ_2 , when the tertiary structure of the PH domain is disrupted by the W⁸⁹⁹A mutation [17], or when two residues of its N-terminal half, Y⁵⁰⁹ and F⁵¹⁰, are replaced by alanine residues, supports an important regulatory role of the split

PH domain. The latter residues have recently been mapped, by NMR titration analysis, to the interface of spPH and SH2c and suggested to be involved in SH2c-mediated autoinhibition [11]. The fact that removal of the two residues is also effective in the absence of almost the entire SH2c domain in PLC $\gamma_2\Delta 19$ is difficult to reconcile with an important role of a spPH-SH2c interaction in cool-temperature regulation of mutant PLC γ_2 . However, chemical shift perturbations observed in NMR experiments probing protein-protein-interactions may imply structural reorientation of residues upon binding rather than direct involvement in the interaction surface [46]. This and the weak interaction between the two domains observed before [32] is consistent with additional functions of these two spPH domain residues. Interestingly, Y⁵⁰⁹ of PLC γ_1 corresponds to Y⁴⁹⁵ of PLC γ_2 , which is mutated to C⁴⁹⁵ in PLC γ_2^{Ali14} and the double mutant PLC $\gamma_2^{\text{Ali5/Ali14}}$. The considerable stimulatory effect of the Ali14 mutation on the stimulation of PLC γ_2^{Ali5} by cool temperature (*cf.* Fig. 7, *left panel*) suggests there may loss- and gain-of function mutations in this locus with regard to cool-temperature sensitivity of PLC γ_2 . Collectively, the results discussed up until this point suggest a new regulatory role of the PLC γ_2 split PH domain in mutant enzyme activation by cool temperatures. However, and quite surprisingly, the results shown in Fig. 10B strongly suggest that the split PH domain is not required as such for cool-temperature-mediated activation of mutant PLC γ_2 .

A model summarizing and schematically conceptualizing the responses of the PLAID PLC γ_2 and their further truncated variants to cooling from 37 °C to 31 °C is shown in Fig. 10C-H. We would like to point out that these schematic views are limited by the fact that they solely rely on measurements of PLC γ_2 enzyme activity and that they await confirmation by direct structural analysis or by other types of independent experimental evidence. Nevertheless, at least for the PLAID PLC γ_2 mutants, our results suggest a dynamic, regulatory role of spPH in controlling the cool temperature response of these mutants. It is tempting to speculate that the interaction of spPH with activated Rac is lost during that response (*cf.* Fig. 9A). Of interest, the spPH domains of PLC γ have been suggested before to undergo dynamic changes leading to formation of intermolecular PH domains regulating agonist-mediated Ca²⁺ entry into cells [47-49], although this has remained somewhat controversial [9].

The marked sensitivity of the two PLAID PLC γ_2 mutants to cool temperatures may explain the gain-of-function symptoms observed in PLAID patients such as cold urticaria [19,20] and skin granulomas [50]. They are likely to be caused by cold-temperature-mediated activation of PLC γ_2 in cutaneous mast cells, neutrophils, and monocytes, where the enzyme plays important roles in Fc ϵ R-, integrin-, and Fc γ R-mediated inflammatory skin reactions [51,52]. Symptomatic allergic and autoimmune disease, occurring in 56 % and 26 % of PLAID patients and potentially precipitated by enhanced basal (37 °C) or cool-temperature-mediated activation of PLC γ_2 in mast and other immune cells, may also fall into this category. The latter may include B cells and dendritic cells. Loss-of-

function symptoms, such as antibody deficiency, recurrent sinopulmonary infection, and symptoms resembling certain forms of common variable immunodeficiency [53], detected in 75 %, 44 %, and 11 %, respectively, of PLAID patients, are more difficult to explain. However, at least in the heterologous expression system used here, the two PLAID mutants appeared to be resistant to stimulation by activation of endogenous EGF receptors, both at cool (31 °C) and physiologic (37 °C) temperatures (Fig. 11). These results suggest that resistance to receptor stimulation may, at least in part, be an intrinsic property of the PLAID mutant enzymes themselves or of components endogenously present in COS-7 cells. This is an important issue, since we expect, but certainly need to prove, that observations reported herein in transfected COS-7 cells will be relevant for, e.g., B cells with endogenous PLC γ_2 expression. As for the B cell context, recent results suggest that the SH2c domain of PLC γ_2 plays a critical role in stabilizing the early BCR signaling complex, such that PLC γ_2 mutants lacking a functional cSH2 domain such as PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ may act in a dominant-negative manner to prevent the formation of stable, signaling-competent BCR clusters consisting of Syk, BLNK, Btk, and PLC γ_2 [37]. Furthermore, association of BCR with the inhibitor of Ca²⁺ signaling, Cbl, was dysregulated in B cells expressing PLC $\gamma_2\Delta 20-22$. These results notwithstanding, it is well known that constant antigen receptor occupancy and signaling, including an increased basal concentration of intracellular Ca²⁺ ([Ca²⁺]_i^{basal}) mediates maintenance of B cell anergy, which is characterized by the persistence in the periphery of B cells unresponsive to immunogen [54-58]. A defect leading to impaired BCR-induced increases in [Ca²⁺]_i has been described for type Ia patients with common variable immunodeficiency (Freiburg classification) and shown to be associated with a reduction in IgD⁺IgM⁺ CD27⁺ class-switched memory B cells, hypogammaglobulinemia, and autoimmune dysregulation [59], all of which are observed in PLAID patients. In normal B cells, anergy is rapidly reversed after dissociation of self antigen, e.g. by using hapten competition, and these cells regain antigen responsiveness [52]. In PLAID B cells, [Ca²⁺]_i^{basal} would be expected to be elevated, either chronically due to constitutively enhanced PLC γ_2 mutant activity at 37 °C or intermittently during passage through cool body regions of the patient, causing reversible reductions of B cell responsiveness. Some of the trans-inhibitory mechanisms observed in anergic B cells, inhibiting the signaling of G-protein-coupled chemoattractant receptors [59], may be operative even in other immune cells, such as neutrophils, potentially contributing to the loss-of-immune-response phenotype, e.g. reduced directed migration, observed in PLAID patients [50]. Anergic mechanisms are also in effect in human mast cells in response to persistent cell activation, changing the cells' set points for further activation [60]. These mechanisms could explain why cold urticaria is not generally observed in PLAID patients in all regions where the temperature on the skin should be low enough to cause mutant PLC γ_2 activation [37]. In human skin, most mast cells are present immediately below the dermo-epidermal junction [61] and are, hence, exposed to temperatures very similar to those

prevailing on the skin. Small negative deviations from the set point temperature developing over short time periods may be the relevant trigger of cold urticarial lesions in PLAID patients.

An interesting question raised by the dramatic functional consequence of the *PLCG2* deletions observed in PLAID patients is whether the genomic regions encompassing exons 19 through 22 are subject to alternative processing of pre-messenger RNA, leading to exclusion of residues encoded by exons 19 and/or 20-22 from the mature PLC γ_2 protein. This appears as an important issue, since almost 90 % of human genes undergo alternative splicing with a minor isoform frequency of 15 % or more, with variations, intraindividually, between tissues and between individuals [62]. Alternative RNA splicing has been shown to be pervasive across immune system lineages [63]. Intriguingly, one of the two changes observed for PLC γ_2 -encoding mRNA in mouse CD19⁺ B cells is skipping of exons 20-22. The marked similarity between the genomic organization of the mouse *Plcg2* and the human *PLCG2* genes raises the possibility that this alteration may also exist in humans to convey cool-temperature-sensitivity to PLC γ_2 in certain cell types and/or particular individuals. Cold environmental temperatures are associated with a number of human disease states, including, e.g., exercise-induced asthma and acute cold-induced coronary events [64,65]. Cold-mediated activation of variant PLC γ_2 in these states, e.g. in mast cells, appears as an intriguing possibility.

4. Conclusions

(i) The two human PLAID PLC γ_2 mutants PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ are strongly (> 100-fold), rapidly, and reversibly activated in intact cells by cooling the cells by only a few degrees. (ii) The underlying mechanism(s) is distinct from a mere loss of SH-region-mediated autoinhibition and dependent on both the integrity and the pliability of the spPH domain. (iii) The results suggest a new mechanism of PLC γ activation with unique thermodynamic features and assign a novel regulatory role to its spPH domain.

Supplementary data to this article can be found online.

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Author contributions

A.S., C.W., M.W., J.H., P.V., J.K., D.F., H.K., and G.H. performed the experiments and analyzed the data. P.G. provided overall direction and wrote the manuscript with input from J.M. and the other authors.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure legends

Fig. 1. The PLC γ_2 deletion mutants PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ are specifically activated by cool temperatures. (A) Domain organization of the PLC γ isoforms. The positions of the two deletions $\Delta 19$ and $\Delta 20-22$ are indicated. *aa*, amino acids. (B) COS-7 cells were transfected with 500 ng each per well of either empty vector (■), or vector encoding either wild-type PLC γ_2 (●), PLC $\gamma_2\Delta 19$ (▼), or PLC $\gamma_2\Delta 20-22$ (▲). Twenty-four hours after transfection, the cells were incubated for 20 h with *myo*-[2- 3 H]inositol at the indicated temperatures and inositol phosphate formation was then determined. The levels of inositol phosphate formation at 37 °C are shown in expanded scale on the right vertical axis (*open symbols*). (C) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in Fig. 1B. Cells from one well each were washed once with 0.2 ml of Dulbecco's PBS and then lysed by addition of 100 μ l of SDS-PAGE sample preparation buffer. The samples were subjected to SDS-PAGE and immunoblotting was performed using an antibody reactive against the c-Myc epitope on PLC γ_2 . (D) The activation of PLC γ_2 deletion mutants by cool temperatures is not explained by changes in cellular levels of inositol phospholipids. COS-7 cells were transfected with 2 μ g each per well of empty vector (*Control*), vector encoding wild-type PLC γ_2 (*WT*), or PLC $\gamma_2\Delta 19$ ($\Delta 19$). Twenty-four hours after transfection, the cells were incubated for a further 20 h in individual incubation chambers with *myo*-[2- 3 H]inositol at the indicated temperatures. The amount of [3 H]inositol present in the culture medium after radiolabeling of the cells (■) and the cellular formation of inositol phospholipids (●) was then determined as described in Experimental Procedures. The data was analyzed by non-linear least square curve fitting to a polynomial second order (quadratic) equation. (E) The expression of wild-type PLC γ_2 and PLC $\gamma_2\Delta 19$ was examined by subjecting cells from one well each to analysis by SDS-PAGE and immunoblotting using antibodies reactive against the c-Myc epitope.

Fig. 2. The activation of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ by subphysiological temperatures occurs after protein synthesis and is reversible. (A) COS-7 cells were transfected with 500 ng each per well of either vector encoding wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, or PLC $\gamma_2\Delta 20-22$. Twenty-four hours after transfection, the cells were incubated for a further 20 h at 31 °C or 37 °C in individual incubation chambers with *myo*-[2- 3 H]inositol, as indicated in the absence or presence of 100 μ g/ml cycloheximide (*CHX*). (B) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in *panel A*. The control samples (*Co.*) were taken at the end of the initial 24-h-transfection phase. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody

reactive against the c-Myc epitope. We note that despite its effect on PLC γ_2 protein abundance, cycloheximide had only a minor effect on the increased formation of inositol phosphates by the two deletion enzymes at 31 °C *versus* 37 °C (*panel A*). We think that this is due to the fact that the upper limits of available phospholipid substrate were reached in this experiment. Hence, the levels inositol phosphate formation in the samples containing the deletion mutants assayed at 31 °C may have exceeded those determined in *panel A* under conditions of unlimited substrate supply. (C) COS-7 cells were transfected with 500 ng each per well of either vector encoding wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, or PLC $\gamma_2\Delta 20-22$. Twenty hours after transfection the cells were pre-incubated for four hours in individual incubation chambers at 31 °C (31 $\rightarrow$ 31; 31 $\rightarrow$ 37) or 37 °C (37 $\rightarrow$ 37). The cells were then incubated for another 20 h with *myo*-[2- 3 H]inositol in individual incubation chambers at 31 °C (31 $\rightarrow$ 31) or 37 °C (31 $\rightarrow$ 37; 37 $\rightarrow$ 37). (D) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in *panel C*. (E) Time course. COS-7 cells were transfected with 500 ng each per well of either vector encoding wild-type PLC γ_2 (●), PLC $\gamma_2\Delta 19$ (▲), or PLC $\gamma_2\Delta 20-22$ (▼). Cells were transfected and radiolabeled and inositol phosphate formation was then determined. At the indicated time points, the cells were shifted from 37 °C to 31 °C and back to 37 °C, respectively. *Open symbols*, incubation at 37 °C throughout. (F) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in *panel E*. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Fig. 3. Determination of the 10-degree temperature coefficients, Q_{10} . The data shown in Fig. 1B on the cool temperature responses of cells expressing wild-type PLC γ_2 (WT), PLC $\gamma_2\Delta 19$ ($\Delta 19$), and PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$) was taken to determine the Q_{10} values of these responses as detailed in Experimental Procedures. The individual temperatures T_i were plotted against $\log_{10}\left(\frac{A_i}{A_{ref}}\right)$, with the maximum activity of PLC $\gamma_2\Delta 19$ at 31 °C chosen as the reference activity A_{ref} and reference temperature, T_{ref} , respectively. The data of the linear components was analyzed by non-linear least square curve fitting to a polynomial first order (straight line) equation. The slopes of the curves of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ were not significantly different by global curve fitting using shared parameters from each other and, between 25 °C and 31 °C, from the slope obtained for wild-type PLC γ_2 (P = 0.9356 and P = 0.2121, respectively).

Fig. 4. Cool-temperature-mediated activation of PLC $\gamma_2\Delta 19$ and PLC $\gamma_2\Delta 20-22$ is distinct from loss of SH-region-mediated autoinhibition. (A) The temperature sensitivities of the PLAID PLC γ_2 deletion mutants are unique. COS-7 cells were transfected with 500 ng each per well of vector encoding either wild-type PLC γ_1 (●), PLC $\gamma_2\Delta 19$ (▽), PLC $\gamma_2\Delta 20-22$ (△) or mutants of PLC γ_1 carrying deletions corresponding to those in PLC γ_2 and referred to as PLC γ_1 " $\Delta 19$ " (▼) and PLC γ_1 " $\Delta 20-22$ " (▲). (B)

The effects of deletions within the SH2n-SH2c-SH3 region on cool temperature regulation of PLC γ_2 are specific. COS-7 cells were transfected with 50 ng each per well of vector encoding either wild-type PLC γ_2 (●), PLC $\gamma_2\Delta 20-22$ (▲), or PLC $\gamma_2\Delta PCI$ (■) (*left panel*), or with 10 ng each per well of vector encoding either wild-type PLC $\gamma_2\Delta SA$ (○), PLC $\gamma_2\Delta SH$ (□), or PLC $\gamma_2\Delta SH2c$ (*open diamonds*), or 50 ng each per well of vector encoding PLC $\gamma_2\Delta PCI$ (■) as a control (*right panel*). The very similar responses of cells expressing PLC $\gamma_2\Delta PCI$ shows that the results shown in the *two panels* are comparable.

Fig. 5. Basal activities of wild-type and mutant PLC γ_2 isozymes at 37 °C. (A) COS-7 cells were transfected with either 500 ng each per well of empty vector (*Co.*, control) or increasing amounts (10 ng, 150 ng, and 500 ng) of vector encoding wild-type PLC γ_2 (*WT*), PLC $\gamma_2\Delta 19$ ($\Delta 19$), PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$), PLC $\gamma_2\Delta PCI$ (ΔPCI), PLC $\gamma_2\Delta SH2c$ ($\Delta SH2c$), PLC $\gamma_2\Delta SH$ (ΔSH), or PLC $\gamma_2\Delta SA$ (ΔSA). Twenty-four hours after transfection, the cells were incubated for 20 h at 37 °C with *myo*-[2-³H]inositol and inositol phosphate formation was then determined. (B) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in *panel A*. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using antibodies reactive against the c-Myc epitope (*upper panel*) or β -actin (*lower panel*).

Fig. 6. The deletions of *PLCG2* exons 19 and 20-22 synergize to promote basal and cool-temperature-mediated activation of PLC γ_2 . *Left panel*, COS-7 cells were transfected as indicated at the abscissa with 500 ng each per well of either vector encoding wild-type PLC γ_2 (*WT*), or 500 ng, 10 ng, or 20 ng of vector encoding PLC $\gamma_2\Delta 19$ ($\Delta 19$), PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$), or PLC $\gamma_2\Delta 19-22$ ($\Delta 19-22$). Twenty-four hours after transfection, the cells were incubated for a further 20 h, as indicated at the abscissa at 31 °C or 37 °C, in individual incubation chambers with *myo*-[2-³H]inositol. Inositol phosphate formation was then determined as described under Experimental Procedures. The significance of differences between means $\pm$ standard errors was assessed by using the unpaired t test with two-tailed P values with or without Welch's correction as appropriate, as contained in GraphPad InStat[®], version 3.10. Two effects are said to be synergistic, if the effect of two components tested in combination is statistically significantly higher than the sum (+) of the individual effects (**, 0.001 < P < 0.01; ***, P < 0.001). *Right panel*, COS-7 cells were transfected with 500 ng of vector encoding wild-type PLC γ_2 (*WT*), PLC $\gamma_2\Delta 19$ ($\Delta 19$), PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$), or PLC $\gamma_2\Delta 19-22$ ($\Delta 19-22$). Twenty-four hours after transfection, the cells were incubated for a further 20 h. Cells from one well each were then analyzed by immunoblotting using an antibody reactive against the c-Myc epitope.

Fig. 7. Effect of cool temperatures on the activities of PLC γ_2^{Ali5} , PLC γ_2^{Ali14} and PLC $\gamma_2^{\text{Ali5/14}}$. *Left panel*, COS-7 cells were transfected with 500 ng each per well of either empty control vector, or vector

encoding either wild-type PLC γ_2 , PLC γ_2^{Ali5} , PLC γ_2^{Ali14} , or PLC $\gamma_2^{\text{Ali5/Ali14}}$. Twenty-four hours after transfection, the cells were incubated for 20 h in individual incubation chambers with *myo*-[2- ^3H]inositol at 31°C or 37°C. *Middle and right panel*, COS-7 cells were transfected as indicated with 500 ng each per well of vector encoding either wild-type PLC γ_2 ($\circ$), PLC $\gamma_2\Delta 20-22$ ($\blacktriangle$), PLC γ_2^{Ali5} ($\blacksquare$), PLC γ_2^{Ali14} ($\blacklozenge$), or PLC $\gamma_2^{\text{Ali5/Ali14}}$ ($\bullet$). Twenty-four hours after transfection, the cells were incubated for 20 h in individual incubation chambers with *myo*-[2- ^3H]inositol at the indicated temperatures. In the *right panel*, cells that had been transfected with vector encoding PLC $\gamma_2^{\text{Ali5/Ali14}}$ were also analyzed as a control. The response of these cells was very similar, both in qualitative and quantitative terms (not shown), to the response of the PLC $\gamma_2^{\text{Ali5/Ali14}}$ -expressing cells shown in the *left* and in the *center panel*, indicating that the results shown in the *three panels* are comparable.

Fig. 8. Functional effects of deletions within the SH2n-SH2c-SH3 region. (A) COS-7 cells were transfected as indicated with 500 ng each per well of empty control vector or vector encoding wild-type PLC γ_2 , or with 10 ng each per well of vector encoding PLC $\gamma_2\Delta\text{SH2n}$, PLC $\gamma_2\Delta\text{SH2c}$, PLC $\gamma_2\Delta\text{SH3}$, PLC $\gamma_2\Delta\text{SH2nSH2c}$, PLC $\gamma_2\Delta\text{SH2cSH3}$, PLC $\gamma_2\Delta\text{SH2nSH3}$, or PLC $\gamma_2\Delta\text{SH}$. Twenty-four hours after transfection, the cells were incubated for a further 20 h in individual incubation chambers at 37 °C or 31 °C. (B) Schematic representation of the deletions within the SH2n-SH2c-SH3 region (aa 515-840). The positions of the two partial repeats detected within the two SH2 domains (KDGTFLVR/RDGAFLIR and GRVQHCRI/GKVKHCRI; SH2n/SH2c, identical residues underlined) and of the various deletions used in this study are indicated.

Fig. 9. Cool-temperature-triggered activation of PLAID PLC γ_2 deletion mutants is mediated by the split PH domain. (A) Activation of PLC $\gamma_2\Delta 19$ by Rac2 $^{\text{G12V}}$ is diminished at cool temperatures. *Left panel*, COS-7 cells were cotransfected with 500 ng each per well of vector encoding either wild-type PLC γ_2 ($\blacksquare, \square$) or 100 ng of vector encoding PLC $\gamma_2\Delta 19$ ($\bullet, \circ$) together with 25 ng per well of either empty vector ($\square, \circ$) or vector encoding Rac2 $^{\text{G12V}}$ ($\blacksquare, \bullet$). There was no effect on inositol phosphate formation upon expression of Rac2 $^{\text{G12V}}$ in the absence of PLC γ_2 isozymes (not shown). *Right panel*, COS-7 cells were transfected with either 500 ng per well of vector encoding wild-type PLC γ_2 ($\square$) or increasing amounts of vector encoding PLC $\gamma_2\Delta 19$ ($\circ$). (B) The split PH domain mediates activation of PLC $\gamma_2\Delta 19$ by cold. COS-7 cells were transfected with 500 ng each per well of vector encoding either wild-type PLC γ_2 , PLC $\gamma_2\Delta 19$, PLC $\gamma_2\Delta 19^{\text{W899A}}$, PLC $\gamma_2\Delta 19\text{-PH12}$, PLC $\gamma_2\Delta 19\text{-PH21}$, PLC $\gamma_2\Delta 19\text{-PH11}$, PLC $\gamma_2\text{-PH11}$, or PLC $\gamma_2\Delta 19\text{-PH11}^{\text{Y509A/F510A}}$. (C) Three-dimensional structures of the split PH domains of PLC γ_1 [2FJL [9]] and PLC γ_2 [2W2X [10]], as analyzed and visualized using the PyMOL Molecular Graphics System. The positions of the residues mutated in (B) and the site of insertion of the SH2n-SH2c-SH3 region in wild-type PLC γ_2 , covalently linked in PLC $\gamma_2\Delta\text{SH}$ are indicated. (D) A functional

spPH domain is required for the temperature sensitivity of PLC $\gamma_2\Delta 19$. COS-7 cells were transfected with 500 ng each per well of vector encoding either wild-type PLC γ_2 , PLC γ_2^{Y495A} , PLC γ_2^{C496A} , PLC $\gamma_2^{Y495A/C496A}$, PLC $\gamma_2\Delta 19$, PLC $\gamma_2\Delta 19^{Y495A}$, PLC $\gamma_2\Delta 19^{C496A}$, or PLC $\gamma_2\Delta 19^{Y495A/C496A}$.

Fig. 10. Cool temperature sensitivity of PLC $\gamma_2\Delta SH$ mutant is restored by expression of the enzyme as two separate chains. (A) COS-7 cells were transfected with 500 ng each per well of either empty vector, 500 ng of vector encoding wild-type PLC γ_2 , 10 ng of vector encoding PLC $\gamma_2\Delta SH$, or 250 ng of vector encoding PLC γ_2 -X^{PHn} or PLC γ_2 -Y^{PHc} (both encompassing the respective halves of the split PH domain), or cotransfected with 250 ng of vector encoding PLC γ_2 -X^{PHn} together with 250 ng of vector encoding PLC γ_2 -Y^{PHc}. (B) The split PH domain is not essential for the cool temperature sensitivity of PLC γ_2 expressed as two separate chains. COS-7 cells were transfected with 500 ng each per well of either empty vector (Co.), or vector encoding wild-type PLC γ_2 (WT), PLC γ_2 -X (X), or PLC γ_2 -Y (Y) (the latter two lacking the respective halves of the split PH domain) (all four vectors at 500 ng per well), or cotransfected with 100 ng each per well of vectors encoding PLC γ_2 -X or PLC γ_2 -Y. PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$) (500 ng vector per well) was analysed for comparison. (C-H) Model of the activation of PLAID PLC γ_2 mutants, exemplified by PLC $\gamma_2\Delta 20-22$, and their deletion mutants by cool temperature. (C) Wild-type PLC γ_2 is autoinhibited in its basal state by constituent(s) of its SH domain region and does not respond to cooling (-). (D) The $\Delta 20-22$ deletion within the SH2n-SH2c-SH3 region causes a reorientation of the SH domain region, allowing the enzyme to be markedly activated (++) by cool temperatures in a process depending on the integrity of spPH. Only minor functional changes are caused by the deletion at 37 °C (not shown). (E) Covalent linkage of the two PH domain halves in the constitutively active (+) deletion mutant PLC $\gamma_2\Delta SA$ prevents further activation by cooling. (F) Disengaging the two spPH halves from the tight and stable covalent linkage allows cool temperature activation of the bipartite enzyme (++). (G and H) Coexpression of fragments X and Y yields enzyme that is constitutively active at 37 °C (at suitable expression levels, not shown) (+). Cooling to 31 °C results in marked further activation (++). Note that cool temperature activation of the bipartite PLC γ_2 mutants occurs in the absence of any of the SH2n-SH2c-SH3 constituents (F and H).

Fig. 11. The PLC γ_2 deletion mutants $\Delta 20-22$ and $\Delta 19$ are resistant to stimulation by EGF. (A) COS-7 cells were transfected with vectors encoding either wild-type PLC γ_2 (WT) (500 ng per well), PLC $\gamma_2\Delta 20-22$ ($\Delta 20-22$), or PLC $\gamma_2\Delta 19$ ($\Delta 19$) (both at 100 ng per well). Eighteen hours after transfection, the cells were incubated for a further 24 h as indicated at either 31 °C or 37 °C with myo-[2-³H]inositol and 10 mM LiCl in the absence of serum and then treated for 60 min at the same temperatures in the presence of 10 mM LiCl with 100 ng/ml EGF, followed by determination of

inositol phosphate formation. Background inositol phosphate formation in response to addition of EGF was determined in parallel on cells transfected with empty vector and subtracted from the individual values, with appropriate consideration of error propagation [66]. Additional experiments showed that the stimulatory effect of EGF on wild-type PLC γ_2 activity was concentration-dependent with half-maximal and maximal effects at approximately 10 ng/ml and 50 ng/ml, respectively, and was almost completely blocked (- 95 %) by the EGFR inhibitor cetuximab (*not shown*).

Figure(s)

Fig. 1-I

Fig. 1-II

A **B** **Fig. 2-I**

C **D** **Fig. 2-II**

E **F** **Fig. 2-III**

Fig. 3

A

Fig. 4-I

B **Fig. 4-II**

A

Fig. 5-I

B

Fig. 5-II

Fig. 6

Fig. 7

A

Fig. 8-I

B

Fig. 8-II

Fig. 9-I

A

Fig. 10-I

B

Fig. 10-II

Fig. 11

C **D** **E** **F** **G** **H**

⊖, ⊕, ⊕⊕: PtdIns(4,5) P_2 hydrolysis

Fig. 10-III

SUPPLEMENTARY MATERIAL

Cool-temperature-mediated activation of phospholipase C- γ_2 in the human hereditary disease PLAID

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Supplementary figures S1-S5

Fig. S1. Expression of wild-type and mutant PLC γ isozyms in Figs. 4A and 4B

Fig. S2. Expression of wild-type and mutant PLC γ isozyms in Fig. 5

Fig. S3. Expression of wild-type and mutant PLC γ isozyms in Fig. 9A

Fig. S4. Expression of wild-type and mutant PLC γ isozyms in Figs. 9B and 9D

Fig. S5. Expression of wild-type and mutant PLC γ_2 isozyms and portions thereof in Fig. 10A

Fig. S6. Expression of wild-type and mutant PLC γ_2 isozyms and portions thereof in Fig. 10A

Fig. S7. Expression of wild-type and mutant PLC γ_2 isozyms in Fig. 11

Supplementary Fig. S1

Fig. S1. (A) Expression of wild-type and mutant PLC γ_1 and PLC γ_2 isozymes in the experiment shown in Fig. 4A. (B) Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in Fig. 4B. Cells from one well each were washed once with 0.2 ml of Dulbecco's PBS and then lysed by addition of 100 μ l of SDS-PAGE sample preparation buffer. The samples were subjected to SDS-PAGE and immunoblotting was performed using an antibody reactive against the c-Myc epitope on PLC γ_1 or PLC γ_2 .

Supplementary Fig. S2

Fig. S2. Expression of wild-type and mutant PLC γ_2 isozymes in the experiment shown in Fig. 5, *left panel*, (A), *center panel*, (B), and *right panel*, (C). Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Supplementary Fig. S3

Fig. S3. Expression of wild-type and mutant PLC γ ₂ isozymes in the experiment shown in Figs. 9A, *left panel*, (A) and 9A, *right panel*, (B). Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Supplementary Fig. S4

Fig. S4. Expression of wild-type and mutant PLC γ 2 isozymes in the experiment shown in Figs. 9B (A) and 9D (B). Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Supplementary Fig. S5

Fig. S5. Expression of wild-type and mutant PLC γ 2 isozymes as well as portions thereof in the experiment shown in Fig. 10A. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Supplementary Fig. S6

Fig. S6. Expression of wild-type and mutant PLC γ_2 isozymes as well as portions thereof in the experiment shown in Fig. 10B. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

Supplementary Fig. S7

Fig. S7. Expression of wild-type and mutant PLCγ₂ isoforms in the experiment shown in Fig. 11. Cells from one well each were analyzed by SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.