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The Phospholipase Cγ_2 Mutants R665W and L845F Identified in Ibrutinib-resistant Chronic Lymphocytic Leukemia Patients Are Hypersensitive to the Rho GTPase Rac2 Protein*

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Mutations in the gene encoding phospholipase C-γ_2 (PLCγ_2) have been shown to be associated with resistance to targeted therapy of chronic lymphocytic leukemia (CLL) with the Bruton's tyrosine kinase inhibitor ibrutinib. The fact that two of these mutations, R665W and L845F, imparted upon PLCγ_2 an ~2–3-fold ibrutinib-insensitive increase in the concentration of cytosolic Ca^{2+} following ligation of the B cell antigen receptor (BCR) led to the assumption that the two mutants exhibit constitutively enhanced intrinsic activity. Here, we show that the two PLCγ_2 mutants are strikingly hypersensitive to activation by Rac2 such that even wild-type Rac2 suffices to activate the mutant enzymes upon its introduction into intact cells. Enhanced "basal" activity of PLCγ_2 in intact cells is shown using the pharmacologic Rac inhibitor EHT 1864 and the PLCγ_2^{F897Q} mutation mediating Rac resistance to be caused by Rac-stimulated rather than by constitutively enhanced PLCγ_2 activity. We suggest that R665W and L845F be referred to as allomorphic rather than hypermorphic mutations of *PLCG2*. Rerouting of the transmembrane signals emanating from BCR and converging on PLCγ_2 through Rac in ibrutinib-resistant CLL cells may provide novel drug treatment strategies to overcome ibrutinib resistance mediated by *PLCG2* mutations or to prevent its development in ibrutinib-treated CLL patients.

Inositol-phospholipid-specific phospholipases C (PLCs)[3] regulate many fundamental functions of normal and neoplastic B cells (1, 2). They catalyze the formation of inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol and, at the same time, decrease the local or general plasma membrane abundance of their substrate, phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) (3). Three members of the six mammalian PLC subfamilies, β, γ, δ, ϵ, ζ, and η, play important roles in B cells as follows: PLCβ_2, PLCβ_3, and PLCγ_2. PLCβ_2 and PLCβ_3 are important in mediating B cell responses to G-protein-coupled chemokine receptors (4). PLCγ_2 serves as a key component of the B cell receptor (BCR) signalosome by interacting with cell surface receptor activation, *e.g.* by antigens (5), cleavage fragments of the third complement component (6), and bacterial, viral, or autoimmunity host DNA (7), and even certain chemokines (8). PLCγ_2 activation results in InsP_3-mediated increases in the concentration of free Ca^{2+}, diacylglycerol-mediated activation of protein kinases C, and changes in transmembrane signaling directly mediated by PtdInsP_2 (9).

Several lines of evidence point to an important contribution of enhanced BCR signaling in the pathogenesis, progression, and/or maintenance of B cell leukemias and lymphomas. For example, leukemic B cells of patients with chronic lymphocytic leukemia (CLL) specifically express a restricted immunoglobulin heavy variable (*IGHV*) gene repertoire, suggesting that CLL development represents an antigen-superantigen-driven process (10). Furthermore, the presence or absence of somatic mutations in rearranged *IGHV* genes determines the clinical course of CLL, with patients carrying mutated *IGHV* genes generally following a more indolent course (10). In CLL, the BCR repertoire is characterized by subsets of closely homologous ("stereotyped") immunoglobulin V(D)J sequences, which are directly involved in antigen binding. This, together with the finding that most malignant B cells thrive only poorly *in vitro*, further supports the notion of a role of antigenic drive in B cell tumorigenicity (11). Recent evidence suggests that, at least in CLL, BCRs also induce cell-autonomous signaling independent of extrinsic antigens that is caused by intra- or inter-BCR interactions (12). Finally, there is evidence for the existence of constitutively activated protein kinases and transcription factors downstream of PLCγ_2 in leukemic B cells of certain CLL cells (13–15). The observation that some of the signaling components upstream of PLCγ_2, such as the protein-tyrosine kinases Syk and Btk, can promote B cell proliferation and/or survival, either along the pathway of normal B cell development or at specific stages following malignant transformation, is well in line with this concept (16–18).

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[3] The abbreviations used are: PLC, inositol-phospholipid-specific phospholipase C; SH2, Src homology 2; PH, pleckstrin homology; spPH, split PH domain; Rac, Ras-related C3 botulinum toxin substrate; BCR, B cell receptor; InsP_3, inositol 1,4,5-trisphosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CLL, chronic lymphocytic leukemia; DH, diffuse B-cell lymphoma (Dbl) homology.

The orally bioavailable irreversible Btk inhibitor ibrutinib has recently undergone a remarkably successful evolution as a second-line treatment of patients with relapsed or refractory CLL or mantle cell lymphoma and as a first-line treatment of patients with CLL carrying a del(17p) or *TP53* mutation (19, 20). Currently, the drug is being evaluated for treatment of other diseases, including other malignancies, autoimmune disease, inflammatory diseases, osteoclast-associated bone diseases, and ischemic stroke (21–26). As is the case for other targeted tumor therapies (27), ibrutinib treatment is characterized, in some cases, by the development of acquired drug resistance (28). Thus, whole-exome sequencing of six CLL patients with late relapses revealed C481S mutations in *BTK* of five patients and three distinct mutations in *PLCG2* of two patients as follows: L845F, R665W, and S707Y in one patient with tumor cells also harboring a *BTK* C481S mutation and *PLCG2* R665W representing the sole mutation in the other patient (29). Although the resistance mechanism conferred by the *BTK* C481S mutation is immediately apparent from the fact that the thiol group of Cys-481 is the site of covalent linkage of ibrutinib to Btk close to its ATP-binding site, the mechanisms of action of the mutations found in *PLCG2* remained less well understood. Whereas S707Y had previously been reported as a constitutively activating mutation in the dominantly inherited human disease APLAID (autoinflammation and PLCγ_2-associated antibody deficiency and immune dysregulation) (30), the R665W and L845F mutants of PLCγ_2 appeared to be functionally normal in reconstituted DT40 chicken B cells in the absence of BCR stimulation, but to mediate moderately enhanced and markedly prolonged ibrutinib-resistant increases in $[Ca^{2+}]_i$ following BCR ligation with anti-IgM (29). Very recent evidence showed Btk-independent activation of the overexpressed R665W PLCγ_2 mutant after B cell receptor engagement in Btk-deficient DT40 cells, suggesting Btk independency of this mutant (31). When the same mutant was expressed in PLCγ_2-deficient DT40 cells containing endogenous wild-type Btk, BCR-mediated PLCγ_2 activation was resistant to ibrutinib, but sensitive to pharmacologic inhibitors of Syk and Lyn. These results suggested the existence of protein-tyrosine kinase mechanisms emanating from BCR and bypassing Btk to activate R665W to mediate ibrutinib resistance even in tumor cells lacking BTK mutations (31).

We have previously shown that PLCγ_2 is specifically activated by Rac GTPases by a mechanism independent of PLCγ_2 tyrosine phosphorylation, but dependent on the direct interaction of activated Rac with the bipartite split PH domain (spPH) juxtaposed between the two halves, *X* and *Y*, of the PLCγ_2 catalytic domain (32, 33). Studies using a Rac-resistant mutant of PLCγ_2, F897Q, reconstituted into PLCγ_2-deficient DT40 B cells recently showed that Rac-mediated stimulation of PLCγ_2 amplifies BCR-mediated Ca^{2+} signaling (34). The fact that failure to proliferate in response to immunoglobulin receptor stimulation had also been observed in mice carrying deletions in all three genes encoding Vav guanine nucleotide exchange factors of Rac GTPases, Vav1, −2, and −3 (35), prompted us to examine, in this work, the effect of the *PLCG2* mutations R665W and L845F on the Rac-PLCγ_2 interaction in intact cells and in a cell-free system *in vitro*. The results show that the two mutations take marked stimulatory effects on this interaction. These stimulatory effects may not only contribute to the mechanism(s) of ibrutinib resistance caused by mutations in *PLCG2* rather than *BTK*, but they also provide novel strategies to tackle ibrutinib resistance in CLL and other debilitating human diseases.

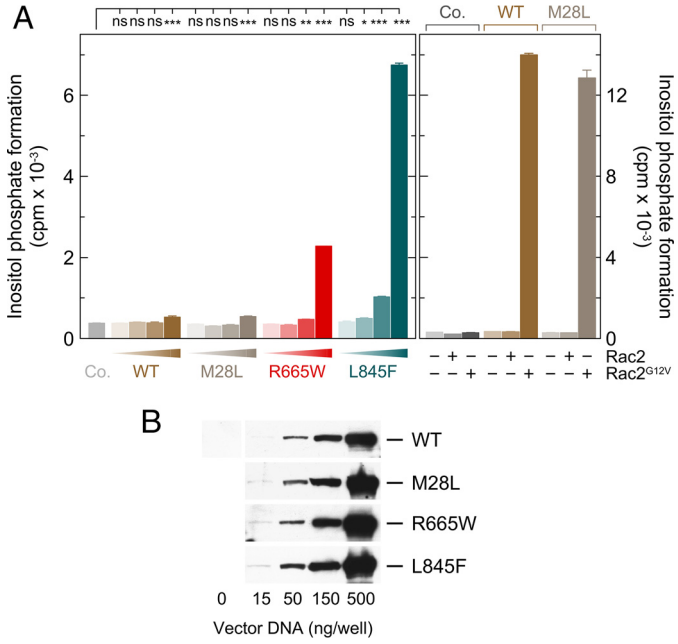

FIGURE 1. **PLCγ_2 point mutations identified in CLL patients differ in their ability to confer enhanced basal activity to the enzyme.** *A, left panel*, COS-7 cells were transfected as indicated with 500 ng/well of either empty vector (*Co.*, control) or increasing amounts (15, 50, 150, and 500 ng/well) of vector encoding either wild-type PLCγ_2 (*WT*), PLCγ_2^{M28L} (*M28L*), PLCγ_2^{R665W} (*R665W*), and PLCγ_2^{L845F} (*L845F*). Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined. *Right panel*, COS-7 cells were cotransfected with 525 ng/well of empty vector (*Co.*) or 500 ng/well of vector encoding either wild-type PLCγ_2 (*WT*) or PLCγ_2^{M28L} (*M28L*), together with 25 ng/well each of either empty vector or vector encoding wild-type Rac2 (*Rac2*) or its constitutively active mutant Rac2^{G12V} (*Rac2^{G12V}*). *B,* homogenates from cells functionally analyzed in *A*, *left panel*, were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ_2.

Results

The first experiment was designed to determine whether the two PLCγ_2 mutants R665W and L845F exhibit constitutive activity in intact cells. To this end, the two mutants were expressed in COS-7 cells to be radiolabeled with [^{3}H]inositol for measurement of [^{3}H]inositol phosphate formation. Wild-type PLCγ_2 and PLCγ_2 carrying an M28L germ line mutation identified in an ibrutinib-resistant patient were analyzed for comparison. Fig. 1*A* shows that, in contrast to wild-type PLCγ_2 and PLCγ_2^{M28L}, the mutants R665W and L845F caused marked, up to 18-fold, increases in basal inositol phosphate formation when expressed in increasing amounts (Fig. 1, *A*, *left panel*, and *B*). Only slight, 1.4-fold, increases were apparent at the highest amounts of wild-type and PLCγ_2^{M28L}. There was no difference between wild-type and M28L mutant PLCγ_2 in terms of their stimulatory responses to constitutively active Rac2^{G12V} (Fig. 1*A*, *right panel*), indicating that PLCγ_2^{M28L} did not harbor a defect in enzyme activation.

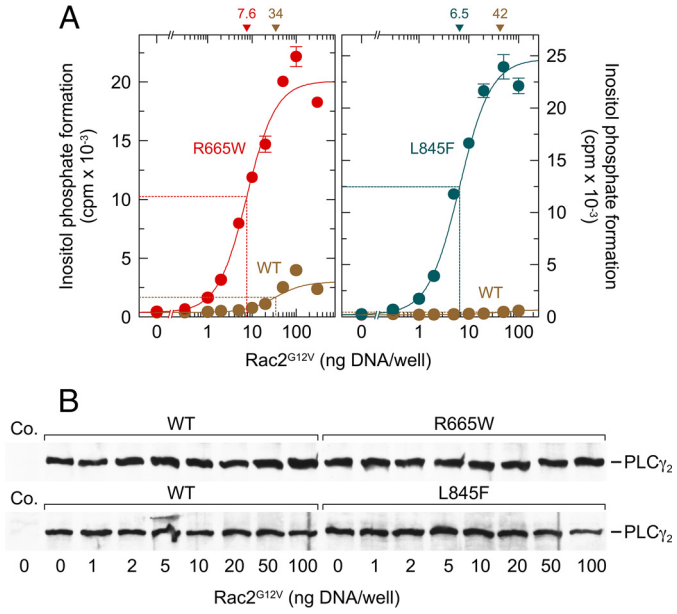

FIGURE 2. **Point mutations R665W and L845F augment the responsiveness of PLCγ_2 to activated Rac2.** *A,* COS-7 cells were transfected as indicated with 150 ng/well vector encoding wild-type PLCγ_2 (*WT*) or PLCγ_2^{R665W} (*R665W*) (*left panel*) or with 50 ng/well vector encoding wild-type PLCγ_2 (*WT*) or PLCγ_2^{L845F} (*L845F*) (*right panel*) and increasing amounts of vector encoding Rac2^{G12V}. Note that the amounts of vector DNA encoding mutant PLCγ_2 was different in the *left* and *right panels* to observe full-range stimulation of the two mutants by Rac2^{G12V} without running out of available phospholipid substrate. Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined. The ED_{50} values of vector encoding Rac2^{G12V} for the stimulation of wild-type or mutant PLCγ_2 activity obtained by non-linear curve fitting are shown *above* the *graphs* in nanograms/well. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope. *Co.,* control.

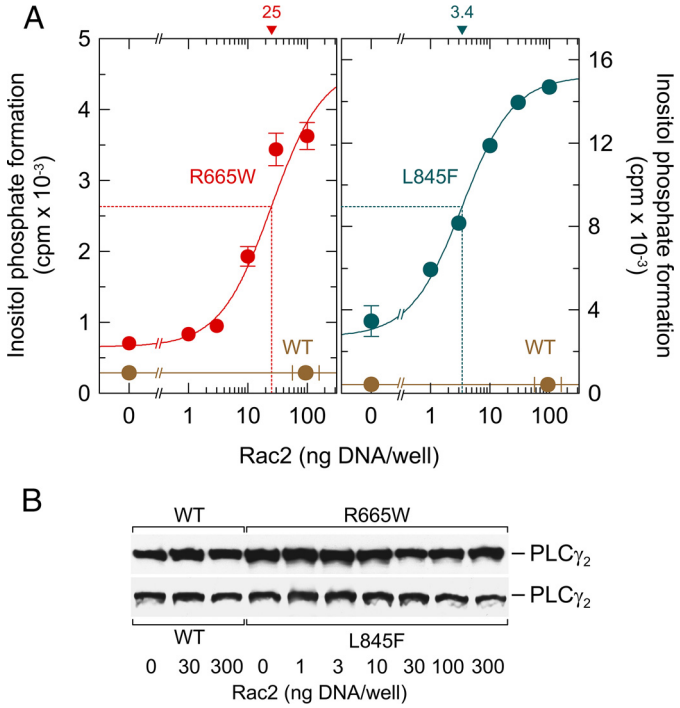

FIGURE 3. **Point mutations R665W and L845F augment the responsiveness of PLCγ_2 to exogenous wild-type Rac2.** *A,* COS-7 cells were transfected as indicated with 500 ng/well vector encoding wild-type PLCγ_2 (*WT*) or PLCγ_2^{R665W} (*R665W*) (*left panel*) or wild-type PLCγ_2 (*WT*) or PLCγ_2^{L845F} (*L845F*) (*right panel*) and increasing amounts of vector encoding Rac2. Note that the vectors encoding mutant PLCγ_2 were used at the same maximal amount (500 ng/well) in the *left* and *right panels* to observe the stimulation by wild-type Rac. Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined. The ED_{50} values of vector encoding Rac2 for the stimulation of mutant PLCγ_2 activity obtained by non-linear curve fitting are shown *above* the *graphs* in nanograms. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

To determine and compare the sensitivity of wild-type PLCγ_2 to stimulation by constitutively active Rac2 to the sensitivities of the mutants R665W and L845F, the PLCγ_2 isozymes were coexpressed with increasing amounts of Rac2^{G12V}. Fig. 2*A* shows that there were striking increases in inositol phosphate formation in response to increasing amounts of Rac2^{G12V}. Specifically, the maximal increase in Rac2^{G12V} efficacy was ~6.7- and 35-fold for PLCγ_2^{R665W} and PLCγ_2^{L845F}, respectively. In addition, we consistently observed that the two point mutations caused an increase in the potency of Rac2^{G12V}, which was ~4.5- and 6.5-fold for PLCγ_2^{R665W} and PLCγ_2^{L845F}, respectively. The increase in Rac2-stimulated PLC activity caused by the PLCγ_2 mutations was not caused by changes in PLCγ_2 protein production in transfected cells (Fig. 2*B*).

Most interestingly, enhanced sensitivity of PLCγ_2^{R665W} and PLCγ_2^{L845F} to Rac2 was not limited to constitutively active Rac2^{G12V} but was also observed for wild-type Rac2 (Fig. 3*A*). Specifically, although there was no effect of increasing amounts of Rac2 on the activity of wild-type PLCγ_2, the mutants R665W and L845F were activated up to 5.1- and 4.2-fold, respectively. There was little, if any, change in the expression of wild-type or mutant PLCγ_2 in the presence of increasing amounts of Rac2 (Fig. 3*B*).

We have previously shown that PLCγ_2 is sensitive to stimulation by exogenous, constitutively active Vav1, Vav1ΔN, presumably via activation of endogenous Rac GTPases present in COS-7 cells (33). Fig. 4*A* shows that the two ibrutinib resistance mutations imparted on PLCγ_2 a marked increase in its responsiveness to stimulation by Vav1ΔN, which amounted to ~9.3- and 12-fold for the mutants R665W and L845F, respectively. As observed for Rac2^{G12V}, there was an increase in the apparent potency of Vav1ΔN by the mutations, which was ~5.3- and 5.7-fold for PLCγ_2^{R665W} and PLCγ_2^{L845F}, respectively. The results shown in Fig. 4*B* suggest that the increase in inositol phosphate formation by wild-type PLCγ_2, but not by its mutants may have, at least in part, been due to a slight increase in the production of the protein at increasing concentrations of Vav1ΔN. The ability of activated Vav1 to catalyze activation of Rho GTPases (36) is dependent on an intact interaction network between the DH, PH, and cysteine-rich domain regions of the Vav1 protein and is abolished by an Asp to Ala mutation at position 376, D376A (37). Fig. 4*C* shows that the inactivating Vav1 mutation caused an almost complete (~85%) and a complete loss in the stimulatory effect of Vav1ΔN on the activity of R665W and L845F, respectively.

The abilities of wild-type and constitutively active Rac2 to cause enhanced activation of PLCγ_2^{R665W} in comparison with wild-type PLCγ_2 were dependent on the C-terminal isoprenylation of the exogenous Rac2 proteins (Fig. 5*A*). Specifically, their stimulatory effect on PLCγ_2^{R665W} activity was completely

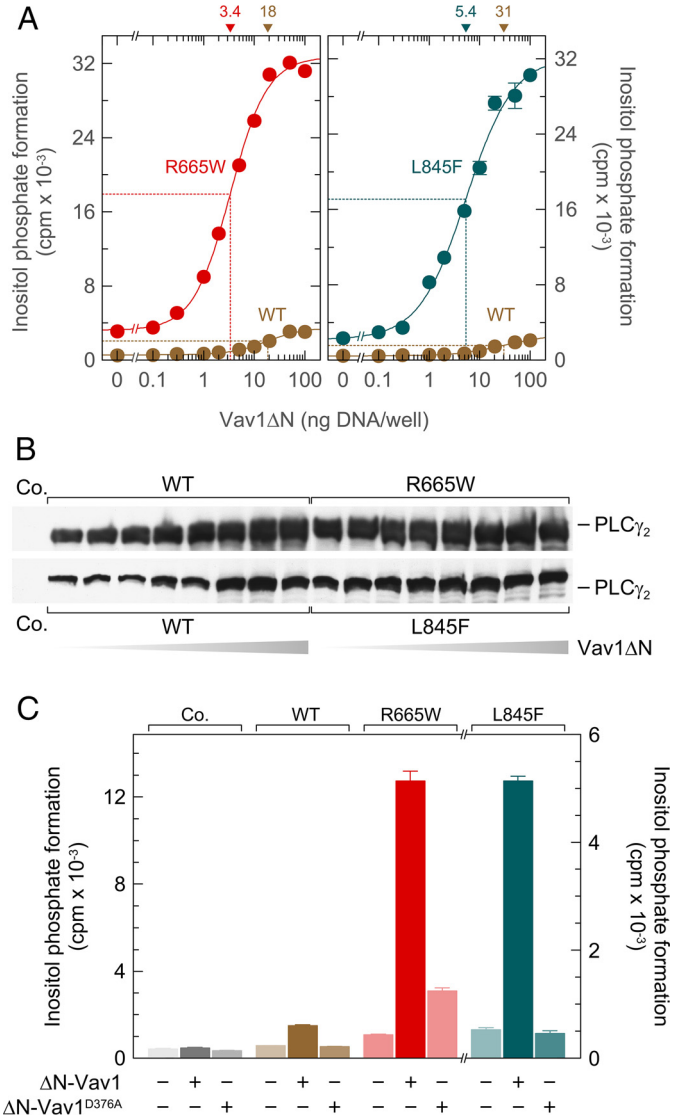

FIGURE 4. **Point mutations R665W and L845F augment the responsiveness of PLCγ_2 to exogenous activated Vav1 even in the absence of exogenous Rac.** *A,* COS-7 cells were transfected as indicated with 500 ng/well vector encoding wild-type PLCγ_2 (*WT*) or PLCγ_2^{R665W} (*R665W*) (*left panel*) or 50 ng/well vector encoding WT PLCγ_2 (*WT*) or PLCγ_2^{L845F} (*L845F*) (*right panel*) and increasing amounts of vector encoding Vav1ΔN. Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined. The ED_{50} values of vector encoding Vav1ΔN for the stimulation of wild-type or mutant PLCγ_2 activity obtained by non-linear curve fitting are shown *above* the *graphs* in nanograms/well. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope. *C,* ability of the point mutations R665W and L845F to enhance stimulation of PLCγ_2 by activated Vav1 is dependent on an intact interaction network between the DH, PH, and cysteine-rich domain regions of the Vav1 protein. COS-7 cells were transfected as indicated with 500 ng each per well of either empty vector (*Co.*, Control), vector encoding wild-type PLCγ_2 (*WT*), or vector encoding PLCγ_2^{R665W} (*R665W*), or 50 ng/well vector encoding PLCγ_2^{L845F} (*L845F*) and 100 ng each per well of vector encoding either Vav1ΔN (*ΔN-Vav1*) or its DH domain mutant Vav1ΔN^{D376A} (*ΔN-Vav1^{D376A}*). Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined.

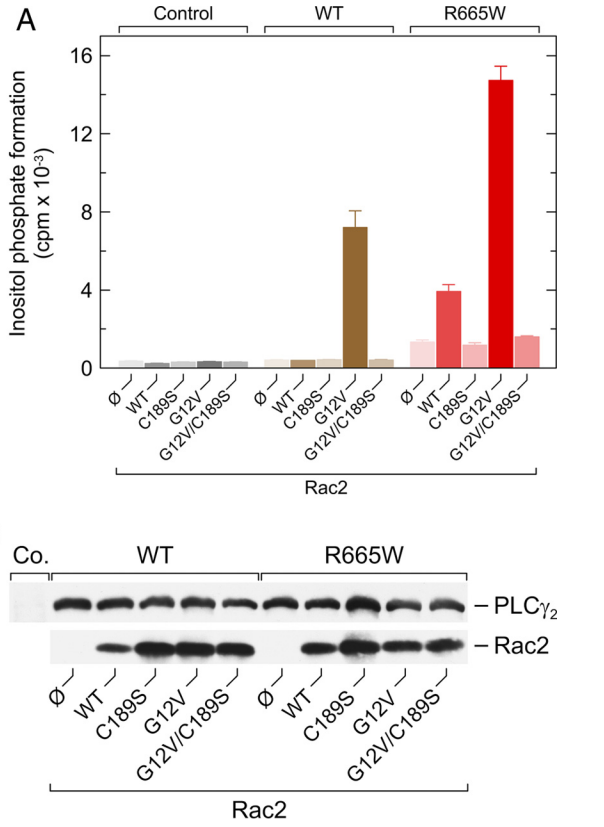

FIGURE 5. **Abilities of exogenous wild-type and constitutively activated Rac2 to activate PLCγ_2^{R665W} are dependent on C-terminal isoprenylation of Rac2.** *A,* COS-7 cells were transfected with 500 ng/well empty vector (*Co.*, control) or vector encoding either wild-type PLCγ_2 (*WT*) or PLCγ_2^{R665W} (*R665W*), together with 10 ng each per well of empty vector (Ø) or vector encoding Rac2 (*WT*), Rac2^{C189S} (*$C^{189}S$*), Rac2^{G12V} (*$G^{12}V$*), or Rac2$^{G12V\,C189S}$ (*$G^{12}V|C^{189}S$*). Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was then determined. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ_2 (*top*) or antiserum reactive against Rac2 (*bottom*).

lost upon replacement of the cysteine residue at position −4 from the Rac2 C terminus, which normally serves as a substrate for geranylgeranylation, by a serine residue, C189S (38). This was despite the fact that the C189S mutants were expressed at similar, if not somewhat enhanced levels, as compared with their respective counterparts (Fig. 5*B*).

Figs. 3*A*, 4, *A* and *C*, and 5*A* also confirm the increase in basal activity of PLCγ_2^{R665W} and PLCγ_2^{L845F} even in the absence of exogenous PLCγ_2 stimuli such as Rac2 and Vav1ΔN observed in Fig. 1. The fact that COS-7 cells are transformed cells prompted us to investigate the following possibilities: (i) that enhanced basal activity is caused by cell-autonomous activation of the mutant PLCγ_2 isoforms, *e.g.* by spontaneously active cell surface receptors, and (ii) that Rac participates in this activation. To this end, F897Q mutants of wild-type PLCγ_2 and its mutants R665W and L845F were generated and functionally characterized (Fig. 6*A*). We have previously shown that the F897Q substitution blocks activation of PLCγ_2 by constitutively active Rac2 and abolishes binding of GTPγS-activated Rac2 to PLCγ_2 spPH, while leaving the overall fold of PLCγ_2 spPH unaffected (33). Fig. 6*A*, *left panel,* shows that the F897Q mutation, as expected, caused a complete or near complete loss of activation of wild-type PLCγ_2 by Rac2^{G12V} and of PLCγ_2^{R665W} by wild-type and G12V mutant Rac2. More importantly, however, the increased "basal" PLCγ_2^{R665W} activity, determined in the absence of exogenous Rac2, was reduced by

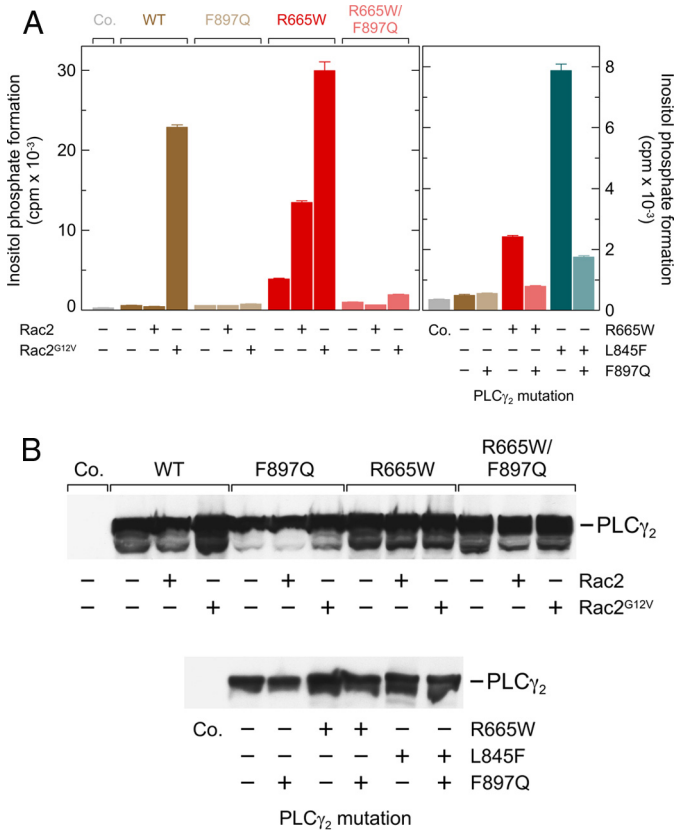

FIGURE 6. **Enhanced Rac2- and Rac2^{G12V}-stimulated activity of PLCγ_2^{R665W} as well as enhanced basal activity of PLCγ_2^{R665W} and PLCγ_2^{L845F} are prevented by a point mutation of PLCγ_2, F897Q, mediating resistance of the enzyme to stimulation by activated Rac2.** *A,* COS-7 cells were transfected with 500 ng/well empty vector (*Co.*, Control) or vector encoding either wild-type PLCγ_2 (*WT*), PLCγ_2^{F897Q} (*F897Q*), PLCγ_2^{R665W} (*R665W*), or PLCγ_2 R665W/F897Q (*R665W|F897Q*), together with 25 ng/well empty vector or vector encoding Rac2 or Rac2^{G12V} (*left panel*). In the *right panel*, COS-7 cells were transfected with 500 ng/well (*from left to right*) empty vector (*Co.*) or vector encoding either wild-type PLCγ_2, PLCγ_2^{F897Q}, PLCγ_2^{R665W}, PLCγ_2 R665W/F897Q, PLCγ_2^{L845F}, or PLCγ_2 L845F/F897Q. Note that the vectors encoding mutant PLCγ_2 were used at the same maximal amount (500 ng/well) to observe the stimulation by wild-type Rac2 (*left panel*) and enhanced basal activity of the PLCγ_2 mutants (*right panel*).Twenty four hours after transfection, the cells were incubated for 20 h with *myo*-[2-^{3}H] inositol, and inositol phosphate formation was determined. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope.

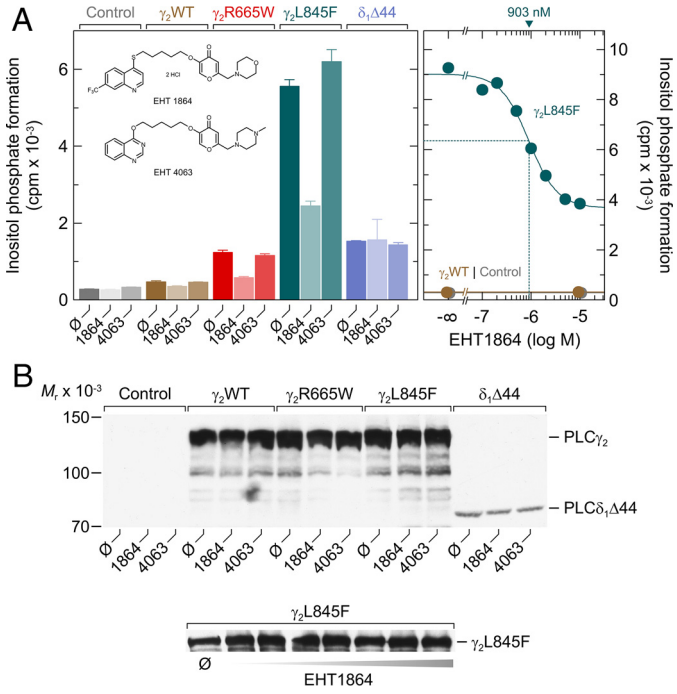

FIGURE 7. **Enhanced basal activities of PLCγ_2^{R665W} and PLCγ_2^{L845F} are specifically reduced by the Rac inhibitor EHT 1864.** *A,* COS-7 cells were transfected with 500 ng/well empty vector (*Control*) or vector encoding wild-type PLCγ_2 (*γ_2WT*), PLCγ_2^{R665W} (*γ_2R665W*), PLCγ_2^{L845F} (*γ_2L845F*), or 30 ng/well of vector encoding PLC$\delta_1\Delta44$ (*$\delta_1\Delta44$*). Twenty four hours after transfection, the cells were incubated for 18 h with *myo*-[2-^{3}H]inositol in the absence (Ø) or presence of 5 μM EHT 1864 or 5 μM of its inactive congener EHT 4063, followed by determination of inositol phosphate formation (*left panel*). The *inset* shows the structural formulas of EHT 1864, 5-(5-(7-(trifluoromethyl)quinolin-4-ylthio)pentyloxy)-2-(morpholino-methyl)-4*H*-pyran-4-one dihydrochloride and EHT 4063, 5-(5-(quinazolin-4-yloxy)pentyl-oxy)-2-((4-methylpiperazin-1-yl)methyl)-4*H*-pyran-4-one. In the *right panel*, COS-7 cells were transfected with 500 ng/well empty vector (*Control*) or vector encoding either wild-type PLCγ_2 (*γ_2WT*) or PLCγ_2^{L845F} (*γ_2L845F*). Twenty four hours after transfection, the cells were incubated for 18 h with *myo*-[2-^{3}H]inositol in the absence or presence of EHT 1864 at the concentrations indicated at the *abscissa*. The IC$_{50}$ value of EHT 1864 for the inhibition of mutant PLCγ_2 activity obtained by non-linear curve fitting is shown *above* the *graph* in nanomolar. *B,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ_2 as well as on PLC$\delta_1\Delta44$.

about 74% (Fig. 6*A*, *left panel*). Fig. 6*A*, *right panel*, shows that F897Q mutation caused similar reductions of basal activity of the R665W and L845F variants of PLCγ_2 (~68 and ~78%, respectively). This indicates that the basal activities of the R665W and L845F mutants are strongly dependent on Rac1 endogenously present in COS-7 cells. These reductions in PLCγ_2 activity were not related to reduced PLCγ_2 protein synthesis in transfected cells (Fig. 6*B*).

To obtain further independent evidence for an involvement of active Rac in the enhanced "basal" activities of PLCγ_2^{R665W} and PLCγ_2^{L845F}, we employed the Rac-specific pharmacologic inhibitor EHT 1864 and its inactive analog EHT 4063 (39). EHT 1864 is known to bind with high affinity to Rac1, Rac1b, Rac2, and with somewhat lower affinity to Rac3. The inhibitor has been suggested to place Rac in an inactive state by promoting the loss of bound guanine nucleotide, rather than interfering with RhoGEF-induced Rac activation, as described for other Rac inhibitors such as NSC23766 (40, 41). Fig. 7*A*, *left panel*, shows that EHT 1864, but not EHT 4063, caused a clear (~55%) inhibition of basal inositol phosphate formation by PLCγ_2^{R665W} and PLCγ_2^{L845F}. There was a smaller (~25%) not quite statistically significant ($p = 0.0676$) inhibitory effect for wild-type PLCγ_2. No effect of EHT 1864 was observed in the absence of exogenous PLC isozyme and in the presence of PLC$\delta_1\Delta44$, a constitutively active variant of PLCδ_1. PLCδ_1 is an evolutionarily divergent relative to PLCγ_2 and insensitive to stimulation by Rac (32, 42). The inhibitory effect of EHT 1864 on basal inositol phosphate formation by PLCγ_2^{L845F} was concentration-dependent with an IC$_{50}$ of about 1 μM (Fig. 7*A*, *right panel*), which is slightly lower than, but still in line with, the previously reported value of about 5 μM for modulation of γ-secretase-mediated amyloid precursor protein (APP) processing (39). There was no effect of EHT 1864 and EHT 4063 on the expression of the various recombinant PLC isozymes in transfected cells (Fig. 7*B*).

The two PLCγ isoforms are distinct in their response to activated Rac, with PLCγ_1, in marked contrast to PLCγ_2, showing

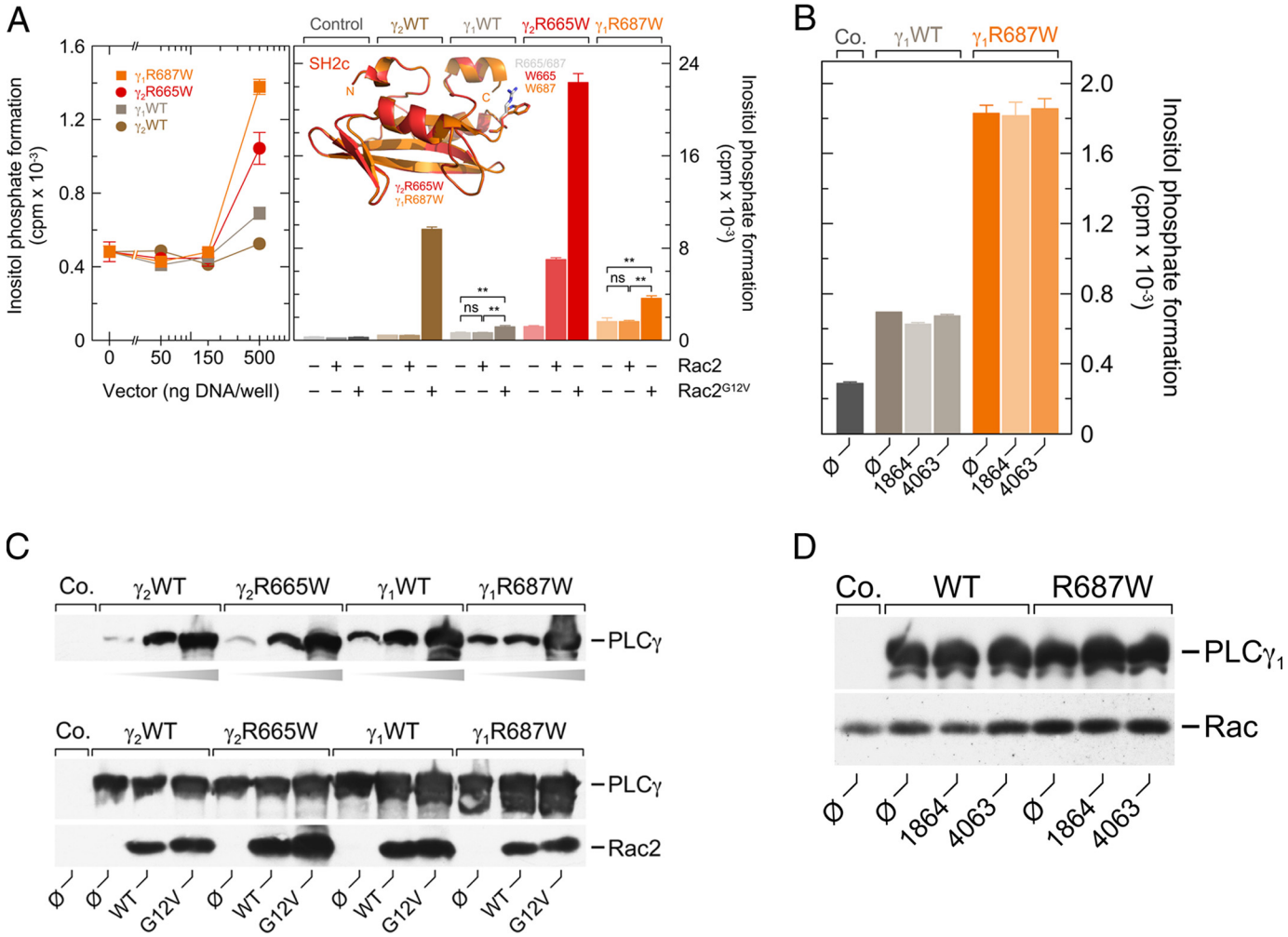

FIGURE 8. **Point mutation in PLCγ$_1$, corresponding to R665W in PLCγ$_2$, R687W, causes enhanced basal activity of PLCγ$_1$ in intact cells that is insensitive to the Rac inhibitor EHT 1864.** *A*, COS-7 cells were transfected with increasing amounts of vector encoding either wild-type PLCγ$_2$ (γ$_2$*WT*), PLCγ$_2^{R665W}$ (γ$_2$*R665W*), PLCγ$_1$ (γ$_1$*WT*), or PLCγ$_1^{R687W}$ (γ$_1$*R687W*) (*left panel*). *Right panel*, COS-7 cells were transfected with 500 ng/well empty vector (*Control*) or vector encoding either wild-type PLCγ$_2$ (γ$_2$*WT*), PLCγ$_2^{R665W}$ (γ$_2$*R665W*), PLCγ$_1$ (γ$_1$*WT*), or PLCγ$_1^{R687W}$ (γ$_1$*R687W*) together with 25 ng/well empty vector or vector encoding Rac2 or Rac2^{G12V}, as indicated at the *abscissa*. The *inset* shows a superimposition of the three-dimensional structures of the C-terminal SH2 domains of PLCγ$_2^{R665W}$ (γ$_2$*R665W*) and PLCγ$_1^{R687W}$ (γ$_1$*R687W*), as predicted by Swiss-Model using the structure of SH2n-SH2c tandem of PLCγ$_1$ as a template (4FBN (67), data not shown) and visualized using the PyMOL Molecular Graphics System. The predicted structures of the wild-type and mutant SH2c domains of PLCγ$_1$ and PLCγ$_2$ and the localizations of the residues in positions 687 (γ$_1$) and 665 (γ$_2$) are virtually identical to each other. Of the two wild-type structures, only the two arginines present in positions 687 (γ$_1$) and 665 (γ$_2$) are shown in *gray*. *B*, COS-7 cells were transfected with 500 ng/well empty vector (*Co.*, control) or vector encoding either wild-type PLCγ$_1$ (γ$_1$*WT*) or PLCγ$_1^{R687W}$ (γ$_1$*R687W*). Twenty four hours after transfection, the cells were incubated for 18 h with *myo*-[2-^{3}H]inositol in the absence (Ø) or presence of 5 μM EHT 1864 or EHT 4063. Subsequently, inositol phosphate formation was determined. *C*, homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ (*top* and *center panels*) or antiserum reactive against Rac2 (*bottom panel*). *D*, homogenates from cells functionally analyzed in *B* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ$_1$ (*top panel*) or antibody reactive against Rac1 (*bottom panel*).

little if any stimulation (32). We therefore set out to examine the functional effects of the PLCγ$_2$ R665W mutation in the PLCγ$_1$ context. The two isozymes are very similar in the overall structure and amino acid sequence in the region close to the point mutation R665W in PLCγ$_2$ and the corresponding residue, Arg-687, in PLCγ$_1$ (*cf.* Fig. 8*A*, *right panel*, *inset*; PLCγ$_1$, LMRVPR^{687}DGAFL; PLCγ$_2$ LMRIPR^{665}DGAFL, single divergent residue underlined). Fig. 8*A*, *left panel*, shows that overexpression of wild-type PLCγ$_1$ and wild-type PLCγ$_2$ led to only minor, if any, changes in basal inositol phosphate formation. Introduction of the point mutations R687W and R665W into PLCγ$_1$ and PLCγ$_2$, respectively, led to no change in activity at lower expression levels. Only at the highest amount of cDNA used for transfection, 500 ng/well, an ~2-fold increase in activity was evident in either case. Fig. 8*A*, *right panel*, shows that neither wild-type nor the R687W mutant PLCγ$_1$ responded to wild-type Rac2, in contrast to the ~5.8-fold enhancement witnessed for the R665W mutation in PLCγ$_2$. Although PLCγ$_1^{R687W}$ exhibited an ~2.3-fold statistically significant ($p < 0.01$) stimulatory response to Rac2^{G12V}, this response was by far less prominent than the ~18.4-fold increase observed for PLCγ$_2^{R665W}$. The enhanced basal activity of PLCγ$_1^{R687W}$ was insensitive to the Rac inhibitor EHT 1864 (Fig. 8*B*), unlike those of the PLCγ$_2$ mutants associated with ibrutinib resistance, PLCγ$_2^{R665W}$ and PLCγ$_2^{L845F}$ (*cf.* Fig. 7*A*), indicating that the basal activity of PLCγ$_1^{R687W}$ is independent of Rac activity, despite its low but statistically significant sensitivity to exogenous Rac2^{G12V}. The latter was also evident, as described before

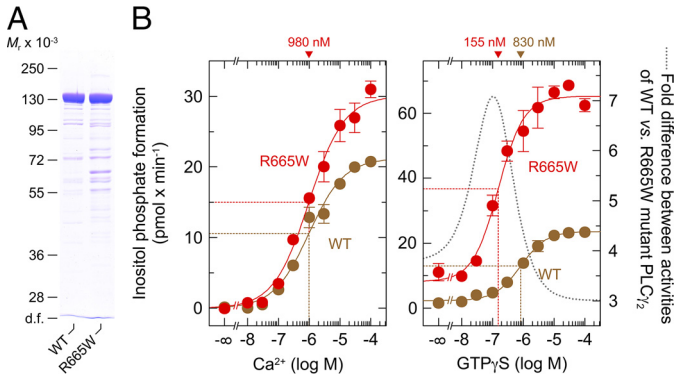

FIGURE 9. **Purified PLCγ_2^{R665W} displays a slight enhancement of basal activity and a marked increase in the sensitivity to purified Rac2 in a reconstituted system *in vitro*.** *A,* recombinant wild-type PLCγ_2 and PLCγ_2^{R665W} were purified from baculovirus-infected insect cells. Aliquots of the two purified preparations were analyzed by label-free quantitative mass spectrometry to determine the relative abundance of PLCγ_2-specific tryptic peptides in the two purified protein preparations. Samples adjusted to contain the same quantities of wild-type and R665W mutant PLCγ_2 were subjected to SDS-PAGE and Coomassie Blue staining. *B,* aliquots of the two samples analyzed in *A* containing equal quantities of purified recombinant wild-type PLCγ_2 and PLCγ_2^{R665W} were incubated at increasing concentrations of free Ca^{2+} and 2.5 mM sodium deoxycholate with phospholipid vesicles containing [^{3}H]PtdInsP_2. There was no difference between wild-type and R665W mutant PLCγ_2 in the concentrations of free Ca^{2+} required to observe half-maximal stimulatory effects (*left panel*). The EC_{50} value of Ca^{2+} for the stimulation of wild-type or mutant PLCγ_2 activity obtained by non-linear curve fitting is shown *above* the *graphs* in nanomolar. In the *right panel*, equal quantities of purified recombinant wild-type PLCγ_2 and PLCγ_2^{R665W} were reconstituted with purified Rac2 in the presence of 30 nM free Ca^{2+} and 1 mM sodium deoxycholate (32) and incubated at increasing concentrations of GTPγS, as indicated at the *abscissa,* with phospholipid vesicles containing [^{3}H]PtdInsP_2. The EC_{50} values of GTPγS for the stimulation of wild-type or mutant PLCγ_2 activity obtained by non-linear curve fitting is shown *above* the *graphs* in nanomolar.

(32), for wild-type PLCγ_1 (Fig. 8*A*, *right panel*). Hypersensitivity to protein tyrosine phosphorylation offers a possible explanation for the increased basal activity of PLCγ_1^{R687W}. Fig. 8*C* shows that although there were noticeable differences in the expression levels of the PLCγ_1 *versus* PLCγ_2 isozymes, in particular at low transfection levels, these differences did not explain the marked functional differences observed in Fig. 8*A*, *right panel*. There were no effects of the EHT compounds on the expression of wild-type or R687W mutant PLCγ_1 or endogenous Rac in the experiment shown in Fig. 8, *B* and *D*.

Next, wild-type and R665W mutant PLCγ_2 were produced as recombinant polypeptides in baculovirus-infected insect cells and purified to near homogeneity by sequential column chromatography (Fig. 9*A*). The two purified preparations were adjusted to contain the same amounts of PLCγ_2 by label-free quantitative mass spectrometry and then used for cell-free determination of inositol phosphate formation from artificial lipid vesicles containing radiolabeled PtdInsP_2 as a substrate. Fig. 9*B*, *left panel*, shows that wild-type and R665W mutant PLCγ_2 displayed a similar dependence on free Ca^{2+} for PtdInsP_2 hydrolysis under these conditions, with half-maximal and maximal hydrolysis occurring at ~1 and 20 μM free Ca^{2+}, respectively. Maximal activity was slightly (~1.4-fold) higher for PLCγ_2^{R665W} than for the wild-type enzyme. Upon functional reconstitution of the two PLCγ_2 isoforms with isoprenylated Rac2 that had also been produced in and purified to near homogeneity from baculovirus-infected insect cells, the R665W mutant PLCγ_2 exhibited a response to increasing concentrations of the poorly hydrolysable Rac2-activating guanine nucleotide analog GTPγS that was clearly different from that of its wild-type counterpart. Specifically, GTPγS showed a higher potency (EC_{50} ~155 nM *versus* ~830 nM) and a higher efficacy (stimulation by ~57 *versus* ~21 pmol inositol phosphates × min^{-1}) to activate the R665W mutant PLCγ_2 in comparison with its wild-type counterpart. Thus, functional differences between wild-type and R665W mutant PLCγ_2 were particularly striking at limited activation of Rac2. A maximal, almost 7-fold difference in Rac2-stimulated activity was observed between wild-type and R665W mutant PLCγ_2 at about 100 nM GTPγS (*cf. dotted lined* in Fig. 9*B*, *right panel*).

COS-7 cells exhibit endogenous expression of EGF receptors, known to be coupled to activation of several intracellular signaling intermediates, including Rac (43). Upon heterologous expression of PLCγ_2 in COS-7 cells, the enzyme is phosphorylated at tyrosine residues and translocated to the plasma membrane to mediate enhanced PtdInsP_2 hydrolysis (44, 45). These previous findings led us to compare the activation of wild-type and L845F mutant PLCγ_2 by endogenously expressed EGF receptors and to examine the relative contribution of tyrosine phosphorylation-mediated and Rac-mediated activation by also studying the two PLCγ_2 isoforms carrying either replacements of four tyrosines known to be phosphorylated by upstream tyrosine kinases during enzyme activation by phenylalanines (4F) or the F897Q mutation blocking activation by Rac. Fig. 10*A*, *left panel*, shows that there was a concentration-dependent increase of wild-type and L845F mutant PLCγ_2 stimulation by EGF, which was half-maximal at ~13 and 6.9 ng/ml EGF, respectively, and maximal at about 50 ng/ml in both cases. Maximal EGF-stimulated PLCγ_2 activity was about 4.5-fold higher in the presence of PLCγ_2^{L845F} in comparison with wild-type PLCγ_2. The results obtained with the 4F, F897Q, and 4F/F897Q mutants of the two variants suggest that about half of the responses of both wild-type and L845F mutant PLCγ_2 were due to tyrosine phosphorylation- and Rac-mediated activation. Similar findings were obtained for PLCγ_2^{R665W} and its F897Q variant. Interestingly, PLCγ_2^{L845F} was sensitive to activation by EGF even in the additional presence of the 4F and F897Q mutations. Wild-type and mutant PLCγ_2 isozymes were present at equal amounts throughout the experiment shown in Fig. 10*A*, *left panel,* and *B*. The ~12.5-fold enhancement of PLCγ_2^{L845F}-mediated inositol phosphate formation by EGF was almost completely blocked (−95%) by the EGF receptor inhibitor cetuximab (Fig. 10*A*, *right panel*).

At least two ibrutinib-resistant patients have been described thus far harboring more than one *PLCG2* mutations, including one with the coexistence of R665W and L845F (46). This prompted us to determine the effects of a compound R665W/L845F mutation on the functions of PLCγ_2. Fig. 11*A* shows that basal activity of PLC$\gamma_2^{R665W/L845F}$ was much higher than that of either PLCγ_2^{R665W} or PLCγ_2^{L845F}, despite similar levels of protein expression (*cf.* Fig. 11*D*). Specifically, when 150 ng of DNA encoding mutant PLCγ_2 was used per well for transfection, the enhancement was ~1.1-, 2.6-, and 70-fold over the activity observed for mock-transfected control cells for PLCγ_2^{R665W}, PLCγ_2^{L845F}, and PLC$\gamma_2^{R665W/L845F}$, respectively. In addition to

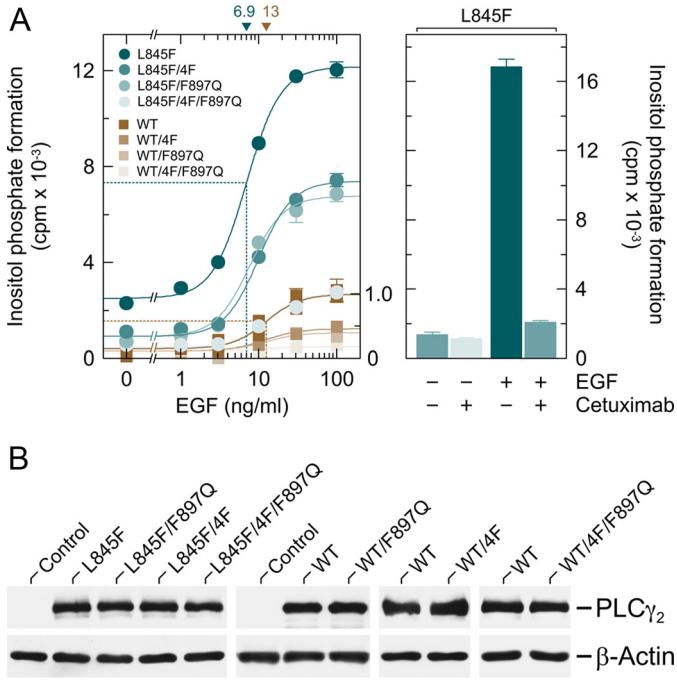

FIGURE 10. **PLCγ_2^{L845F} is hypersensitive to activation by EGF receptor(s) endogenously expressed in COS-7 cells by a mechanism dependent on both protein tyrosine phosphorylation and activation by Rac.** *A*, COS-7 cells were transfected with 150 ng/well vectors encoding either PLCγ_2^{L845F} (*L845F*), PLC$\gamma_2^{L845F/4F}$ (*L845F/4F*), PLC$\gamma_2^{L845F/F897Q}$ (*L845F/F897Q*), or PLC$\gamma_2^{L845F/4F/F897Q}$ (*L845F/4F/F897Q*) or wild-type PLCγ_2 (*WT*), PLCγ_2^{4F} (*WT/4F*), PLCγ_2^{F897Q} (*WT/F897Q*), or PLC$\gamma_2^{4F/F897Q}$ (*WT/4F/F897Q*) (all constructed in pMT2 vector; Tyr → Phe substitutions at amino acid positions 753, 759, 1197, and 1217). Eighteen hours after transfection, the cells were incubated for a further 24 h with *myo*-[2-^{3}H]inositol in the absence of serum and then treated for 60 min in the presence of 20 mM LiCl with increasing concentrations of EGF (1, 3, 10, 30, and 100 ng/ml), followed by determination of inositol phosphate formation. Background inositol phosphate formation in response to addition of EGF at increasing concentrations was determined in parallel on cells transfected with empty vector and subtracted from the individual values, with appropriate consideration of error propagation (68). The data on PLCγ_2^{L845F} and its mutants are from one experiment; the data on wild-type PLCγ_2 and its mutants are from three experiments, each comparing the activity of wild-type PLCγ_2 and one of the mutants. The latter activities were normalized to the maximal activity of PLC$\gamma_2^{L845F/4F/F897Q}$ used as an internal control in one of the latter experiments (shown as a fraction of 1.0 on the *right y* axis). The EC_{50} values of EGF for the stimulation of wild-type or L845F mutant PLCγ_2 activity obtained by non-linear curve fitting are shown *above* the *graphs* in nanograms/ml (*left panel*). In the *right panel*, COS-7 cells were transfected with 150 ng/well vector encoding PLCγ_2^{L845F} (*L845F*) and then incubated as described above and treated for 60 min in the absence or presence of 20 μg/ml cetuximab without or with 10 ng/ml EGF in medium containing 20 mM LiCl prior to determination of inositol phosphate formation. *B*, homogenates from cells functionally analyzed in *A*, *left panel*, were subjected to SDS-PAGE and immunoblotting using an antiserum reactive against PLCγ_2 or antibody reactive against β-actin.

basal phospholipase C activity, Rac2-, Rac2^{G12V}-, and Vav1ΔN-stimulated activity was also markedly enhanced for PLC$\gamma_2^{R665W/L845F}$ in comparison with the other two mutants (Fig. 11, *B* and *C*). For example, inositol phosphate formation in the presence of Vav1ΔN was enhanced ~1.2-, 3.1-, 9.9-, and 61-fold for wild-type PLCγ_2, PLCγ_2^{R665W}, PLCγ_2^{L845F}, and PLC$\gamma_2^{R665W/L845F}$ when compared with expression of Vav1ΔN alone (Fig. 11*C*, *right panel*). The compound mutation also markedly enhanced the apparent potency of Rac2^{G12V} and Vav1ΔN to activate PLCγ_2. Thus, half-maximal activation was observed for PLC$\gamma_2^{R665W/L845F}$ at about 10–20-fold lower transfection levels of Rac2^{G12V} and Vav1ΔN than for either of the single mutants, PLCγ_2^{R665W} or PLCγ_2^{L845F} (Fig. 11*C*).

Discussion

The functions of PLCγ_2 mutants mediating ibrutinib resistance in CLL patients have previously mostly been characterized following their reconstitution into PLCγ_2-deficient DT40 B cells (29). Even in those cells, expressing only the mutant PLCγ_2 rather than a combination of wild-type and mutant PLCγ_2 isozymes, there was no evidence of autonomous PLCγ_2 signaling. Instead, the PLCγ_2 mutants R665W and L845F still relied on BCR activation. The main change was an ~2–3-fold increase in the level of cytosolic Ca^{2+} upon BCR ligation, which was insensitive to inhibition by ibrutinib and, interestingly, did not return to baseline within the time frame of the experiment. Subsequent experiments in reconstituted DT40 cells showed that BCR-mediated activation of PLCγ_2^{R665W} was enhanced even in Btk$^{-/-}$ cells in comparison with the wild-type enzyme, suggesting that the mutant functionally bypasses Btk upon BCR activation (31). In cells expressing PLCγ_2^{R665W}, the BCR-mediated increase in Ca^{2+} was sensitive to pharmacologic inhibitors of Syk and Lyn, signaling components previously known to be essential for BCR-mediated InsP_3 generation and rapid Ca^{2+} mobilization, respectively, in DT40 cells (47). These results suggested that the R665W mutation renders PLCγ_2 independent of Btk and therefore capable of mediating ibrutinib resistance in CLL cells.

Using the same experimental model, reconstituted PLC$\gamma_2^{-/-}$ DT40 cells, we have previously shown that interaction of PLCγ_2 with Rac amplifies the BCR-induced Ca^{2+} signaling by increasing the sensitivity of the cells to BCR ligation, augmenting the BCR-mediated Ca^{2+} release from intracellular stores, enhancing the Ca^{2+} entry from the extracellular compartment, and facilitating the nuclear translocation of the Ca^{2+}-regulated nuclear factor of activated T cells (34). Although performed in a different cellular system, the results presented here suggest that the bypass of Btk exploited by the PLCγ_2 mutant R665W may also be based on an increased sensitivity of this and another mutant, L845F, to enhanced activation by Rac.

Our results showing marked increases in basal inositol phosphate formation upon expression of the two PLCγ_2 mutants R665W and L845F at increasing levels, suggest, at first glance, that the mutants exhibit constitutively enhanced intrinsic activity. Given that constitutive protein activity is a hallmark of hypermorphic mutations (48), this is consistent with the designation of the two PLCγ_2 mutations as belonging to the hypermorphic class (31). However, several lines of evidence presented in this work suggest that enhanced constitutive PLC activity, such as that observed for other mutants of several PLC isozymes (42), including PLCγ_2 (49), is unlikely to be the main molecular mechanism of ibrutinib resistance. Specifically, both mutants are strikingly hypersensitive to activation by Rac2 and its upstream regulator Vav1, such that even wild-type Rac2 suffices to activate the mutant enzymes, but not their wild-type counterpart, upon its introduction into intact COS-7 cells (Fig. 3*A*). This view is strongly supported by the fact that enhanced basal activity of the mutant enzymes is markedly reduced by the F897Q mutation of PLCγ_2 (Fig. 6*A*), which has previously been shown to block PLCγ_2 activation by Rac but not by loss of SH-

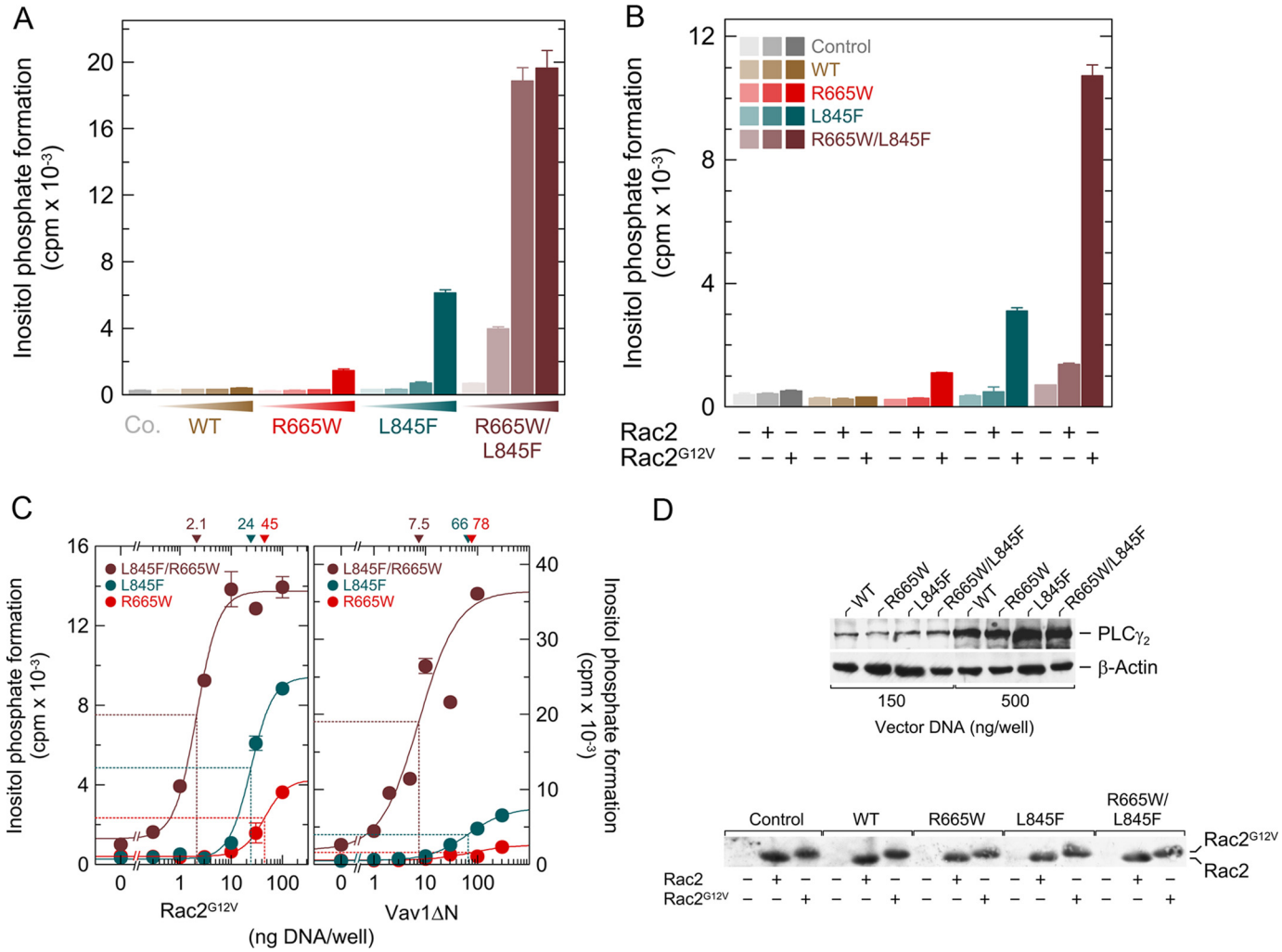

FIGURE 11. **PLCγ_2 ibrutinib resistance mutations R665W and L845F synergize to enhance basal enzyme activity and to sensitize the enzyme to stimulation by activated Vav1 and Rac2.** *A*, COS-7 cells were transfected either with 500 ng/well empty vector (*Co.*, control) or increasing amounts (15, 50, 150, and 500 ng/well) of vector encoding either PLCγ_2 (*WT*), PLCγ_2^{R665W} (*R665W*), PLCγ_2^{L845F} (*L845F*), or the compound mutant PLC$\gamma_2^{R665W/L845F}$ (*R665W/L845F*). *B*, COS-7 cells were transfected with 15 ng/well either empty vector (*Co.*, *Control*) or vector encoding either PLCγ_2 (*WT*), PLCγ_2^{R665W} (*R665W*), PLCγ_2^{L845F} (*L845F*), or the compound mutant PLC$\gamma_2^{R665W/L845F}$ (*R665W/L845F*), together with 25 ng/well empty vector or vector encoding Rac2 or Rac2^{G12V}. *C*, *left panel*, COS-7 cells were transfected as indicated with 15 ng/well vector encoding either PLCγ_2^{R665W} (*R665W*), PLCγ_2^{L845F} (*L845F*), or PLC$\gamma_2^{R665W/L845F}$ (*R665W/L845F*) and increasing amounts of vector encoding Rac2^{G12V}. *Right panel*, COS-7 cells were transfected with 50 ng/well vector encoding either PLCγ_2^{R665W} (*R665W*), PLCγ_2^{L845F} (*L845F*), or PLC$\gamma_2^{R665W/L845F}$ (*R665W/L845F*) and increasing amounts of vector encoding Vav1ΔN. The ED_{50} values of vector encoding Rac2^{G12V} or Vav1ΔN for the stimulation of mutant PLCγ_2 activity obtained by non-linear curve fitting are shown *above* the *graphs* in nanograms/well. In all panels, the cells were incubated 24 h after transfection for 20 h with *myo*-[2-^{3}H]inositol, and inositol phosphate formation was determined. *D, upper panel,* homogenates from cells functionally analyzed in *A* were subjected to SDS-PAGE and immunoblotting using an antibody reactive against the c-Myc epitope present on wild-type and mutant PLCγ_2 or antibody reactive against β-actin. *Lower panel*, homogenates from cells functionally analyzed in *B* were subjected to SDS-PAGE and immunoblotting using an antiserum reactive against Rac2.

mediated autoinhibition or Ca^{2+} (34). Further support stems from the observations that this activity is subject to specific inhibition by the small molecule Rac inhibitor EHT 1864 (Fig. 7*A*) and that the purified PLCγ_2^{R665W} displays only a subtle increase of its basal activity (Fig. 9*B*). Hence, it appears likely that the mutants are hypersensitive to activated Rac2 rather than simply constitutively active.

Several lines of evidence have been presented suggesting that Rac is activated by BCR ligation. Thus, several elements of the canonical BCR signaling cascade, *e.g.* Syk (50), Btk (51), and BLNK (52, 53), are known to physically interact with and activate the Rac activator Vav, by processes not necessarily involving the protein kinase activity of Btk. BCR cross-linking caused activation of both Rac1 and Rac2 within minutes (54). Total internal reflection fluorescence microscopy has shown that PLCγ_2, Vav, BLNK, and Btk synergize to form highly coordinated microsignalosomes. Very interestingly, efficient assembly of the latter is absolutely dependent on Lyn and Syk (55). Therefore, it appears likely that one of the major functional consequences of the two PLCγ_2 mutations conferring ibrutinib resistance to intact cells, including B lymphocytes, is hypersensitivity of PLCγ_2 to activated Rac. The finding that the enhanced PLCγ_2 stimulation by ligation of endogenous EGF receptors requires the replacement by phenylalanines of the tyrosine residues involved in enzyme activation in addition to a F897Q mutation to be maximally reduced (Fig. 10*A*, *left panel*) suggests that tyrosine phosphorylation may be involved, in addition to direct PLCγ_2-Rac interaction in mediating hyper-

sensitivity of PLCγ_2 to Rac. In intact B cells, this hypersensitivity is likely to be the molecular basis of a relatively focused rewiring of the signaling pathways immediately downstream of the BCR, such that PLCγ_2 loses its dependence on activated Btk (56) and gains sensitivity to the pathway made up of Lyn, Syk, Vav, and Rac. Hence, in our opinion, the two PLCγ_2 ibrutinib resistance mutations, R665W and L845F, are not simply and solely hypermorphic. We suggest that they would be better termed allomorphic, according to the Greek word $\alpha\lambda\lambda o\sigma$ for other, different.

In B cells, activation of Rac is not limited to BCR activation, but also occurs upon activation of BCR coreceptors, such as CD19/CD21, integrins, as well as certain G-protein-coupled chemokine and Toll-like receptors (*cf.* discussion and references in Ref. 34). It is thus possible that the rewiring process induced by the ibrutinib resistance mutations also enhances the sensitivity of PLCγ_2 to extracellular ligands of these cell surface proteins, such as cleavage fragments of the third complement component, pathogen-derived molecules, extracellular matrix proteins, and chemokines. This may ultimately allow costimulatory signals to become stimulatory in their own right and as such to alter the interactions of the ibrutinib-resistant tumor cells with their protective microenvironments, for example (57). Interestingly, integrin-mediated adhesion and migration in response to the chemokines CXCL12 or CXCL13, as well as *in vivo* homing to lymphoid organs, was impaired in Btk-deficient (pre-)B cells, whereas CXCL12-mediated activation of Rac was intact. Deficiency of PLCγ_2 also curtailed the CXCL12-mediated migratory response (8). Independence of Btk and increased sensitivity of the PLCγ_2 mutants to Rac by rewiring may provide CLL cells with the ability to home to and remain in protective microenvironments for survival and expansion even in the presence of ibrutinib-mediated Btk inhibition.

Enhanced sensitivity of PLCγ_2 by signaling mechanisms emanating from BCR or other B cell surface receptors bypassing Btk may provide novel mechanisms for targeted treatment of CLL and, possibly, B cell lymphomas. Thus, inhibitors of Syk and Lyn have been shown to oppose ibrutinib resistance mediated by *PLCG2* mutations (31). Although the exact position of PI3Kδ relative to other components within the BCR signalosome is still controversial, some evidence puts class IA PI3Ks, at least in part, upstream of Vav and Rac (58). Hence, inhibition of PI3Kδ with idelalisib may also interfere with Rac-mediated activation of wild-type and, even more so, R665W or L845F mutant PLCγ_2 isozymes. Pharmacologic interventions at the level of Rac itself or other upstream activators also appear to be a viable option. These include inhibition of Rac C-terminal modification or of Rac protein-protein interaction, *e.g.* by small molecules like EHT 1864, or prevention of integrin, CD19, or chemokine receptor activation (59–61). Very recent results suggest that ibrutinib therapy of CLL patients favors selection and expansion of rare subclones already present before ibrutinib treatment, including subclones containing mutations in *PLCG2* (62). Hence, it may be worthwhile to investigate combining ibrutinib with adjuvant drugs of this type *ab initio* to suppress this selection and expansion.

Experimental Procedures

Materials—The mouse monoclonal antibody 9B11 reactive against the c-Myc epitope (EQKLISEEDL) and the rabbit polyclonal antiserum reactive against human PLCγ_2 (sc-407) were obtained from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The rabbit polyclonal antiserum reactive against human Rac2 (sc-96) was purchased from Santa Cruz Biotechnology. The anti-β-actin antibody (clone AC-15) and the anti-Rac1 antibody (clone 23A8) were obtained from Sigma and Merck Millipore, respectively. The Rac inhibitor EHT 1864 and its inactive analog EHT 4063 were synthesized as described previously (63). Human epidermal growth factor (EGF) (E9644) was from Sigma. ProGreen baculovirus vector DNA (A1) was purchased from AB Vector. GTPγS (catalog no. 10220647001) was purchased from Roche Applied Bioscience. Cetuximab is marketed by Merck.

Construction of Vectors—The construction of complementary DNAs encoding c-Myc epitope-tagged human PLCγ_1 (1291 amino acids, accession number ABB84466), human PLCγ_2 (1265 amino acids, accession number NP_002652), and F897Q mutant of PLCγ_2 was described previously (34). The construction of all other vectors and of the baculoviruses was outlined in Refs. 32, 33. Complementary DNAs encoding mutants of PLCγ_1 and PLCγ_2 were constructed by *in vitro* mutagenesis using the QuikChange II XL site-directed mutagenesis kit (200521, Agilent Technologies). The primer sequences and PCR protocols are available from the authors upon request. A vector encoding c-Myc epitope-tagged human PLC$\delta_1\Delta44$ was kindly supplied by J. Sondek (42).

Cell Culture and Transfection—COS-7 cells were maintained at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (catalog no. 41965-039, Gibco) supplemented with 10% (v/v) fetal calf serum (catalog no. 10270-106, Gibco), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories). Prior to transfection, COS-7 cells were seeded into 24-well plates at a density of 0.75×10^5 cells/well and grown for 24 h in 0.5 ml of medium/well. For transfection, plasmid DNA (500–800 ng/well) was diluted in 50 μl of jetPRIME® buffer, and 1–1.6 μl of jetPRIME® was added according to the manufacturer's instructions. The total amount of DNA was maintained constant by adding empty vector. Four hours 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% CO_2.

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 Dulbecco's PBS (PAA Laboratories) and then incubated for 18 h in 0.2 ml/well DMEM containing supplements as described above, supplemented with 2.5 μCi/ml *myo*-[2-^{3}H]inositol (NET1156005MC, PerkinElmer Life Sciences) and 10 mM LiCl. The cells were then washed once with 0.2 ml/well of Dulbecco's PBS and lysed by addition of 0.2 ml/well 10 mM ice-cold formic acid. The analysis of inositol phosphate formation was performed as described previously (33).

To examine EGF-mediated PLCγ_2 stimulation, COS-7 cells were radiolabeled for 24 h in serum-free DMEM as described previously (45). Briefly, cells were washed twice with 0.3 ml/well DMEM containing the above supplements except serum and then incubated for 24 h in 0.2 ml/well of the same medium supplemented with 0.25% fatty-acid-free bovine serum albumin (catalog no. A8806, Sigma) and 2.5 μCi/ml *myo*-[2-^{3}H]inositol. The cells were then washed with 0.3 ml/well Dulbecco's PBS and incubated for 1 h in 0.2 ml/well DMEM without serum containing the above supplements, 20 mM LiCl, and increasing concentrations of EGF. After removal of the medium, the cells were lysed by addition of 0.2 ml/well of 10 mM ice-cold formic acid for analysis of inositol phosphate formation.

Expression and Purification of Proteins—Post-translationally modified Rac2 was expressed as a glutathione *S*-transferase fusion protein in baculovirus-infected insect cells and solubilized from the particulate fraction. The Rac2 portion of the fusion protein was purified as detailed previously (33). c-Myc epitope-tagged PLCγ_2 and PLCγ_2^{R665W} were purified from soluble fractions of baculovirus-infected High Five™ insect cells grown in suspension culture by sequential chromatography on HiTrap™ Heparin HP and Resource Q (GE Healthcare) as described before for PLC$\beta_2\Delta$ (64). Our attempts at purifying PLCγ_2^{L845F} for functional analysis have thus far been unsuccessful, mostly due to the lower expression of the enzyme in baculovirus-infected insect cells and to its lower stability during purification.

Label-free Quantitative Mass Spectrometry—Equal volumes (5 μl each) of purified wild-type or R665W mutant PLCγ_2 were mixed with 300 fmol of the Pierce™ peptide retention time calibration mixture (PRTC, catalog no. 88320, ThermoFisher) containing 15 known synthetic tryptic peptides. Samples were reduced for 20 min at room temperature with 5 mM DTT and subsequently alkylated for 20 min at 37 °C with 50 mM iodoacetamide. The samples were then subjected to tryptic digestion overnight at 37 °C. The resulting peptides were identified by LC/MS analysis. Using the SEQUEST search engine within the Proteome Discoverer™ software suite (1.4.1.14, Thermo Scientific), mass spectra were correlated with a database containing the sequences of wild-type and mutant PLCγ_2, a concatenation of the PRTC peptide sequences, and sequences of common contaminants commonly encountered in proteomic experiments, as used in the MaxQuant software (65). For relative quantitation, the precursor ions area detector node within Proteome Discoverer™ was used; preceding event detection was set to 4 ppm.

Measurement of PLC Activity in Vitro—Phospholipase C activity was determined as described (64, 66) with minor modifications. In brief, aliquots (10 μl) of purified PLCγ_2 proteins appropriately diluted in buffer containing 60 mM Tris/maleate, pH 7.3, 84 mM KCl, 3.6 mM EGTA, 2.4 mM dithiothreitol, 2 mg/ml bovine serum albumin were incubated for 45 min at 30 °C in a volume of 60 μl containing 50 mM Tris/maleate, pH 7.3, 70 mM KCl, 3 mM EGTA, 2 mM dithiothreitol, 536 μM phosphatidylethanolamine, 33.4 μM [^{3}H]PtdInsP_2 (185 GBq/mmol), 0.33 mg/ml bovine serum albumin, and the concentrations of sodium deoxycholate and free Ca^{2+} specified in the figure legends. For reconstitution of wild-type and mutant PLCγ_2 with Rac2, purified PLCγ_2 was reconstituted with 5 μl of purified isoprenylated Rac2 and incubated with the phospholipid substrate as described above. The concentration of $CaCl_2$ required to adjust the concentration of free Ca^{2+} to the desired value was calculated using the program EqCal for Windows (Biosoft, Ferguson, MO). The reaction was terminated, and the samples were analyzed for inositol phosphates, as described (64).

Miscellaneous—SDS-PAGE and immunoblotting were performed according to standard protocols using antibodies reactive against the c-Myc epitope for wild-type and mutant PLCγ_2. Immunoreactive proteins were visualized using the ECL Western blotting detection system (GE Healthcare). All experiments were performed at least three times. Similar results and identical trends were obtained each time. Data from representative experiments are shown as means ± S.E. of triplicate determinations. In Figs. 2*A*, 3*A*, 4*A*, 7*A*, 9*B*, 10*A*, and 11*C*, the data were fitted by nonlinear least squares curve fitting to three- or four-parameter dose-response equations using GraphPad Prism®, version 5.04. In certain cases, the global curve fitting procedure contained in Prism® was used to determine whether the best fit values of selected parameters differed between data sets. The simpler model was selected unless the extra sum of squares *F*-test had a *p* value of less than 0.05. Repeated measures analysis of variance with Tukey's post test contained in the GraphPad InStat software package (version 3.10; GraphPad Software, La Jolla, CA) was used for the statistical analysis of the data shown in Figs. 1*A* and 8*A*. Statistically significant effects are denoted by ***, $p < 0.001$; **, $p > 0.001$ and $p < 0.01$; and *, $p > 0.01$ and $p < 0.05$. Non-significant changes are denoted by *ns*, $p < 0.05$.

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