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The Grb2/PLD2 interaction is essential for lipase activity, intracellular localization and signaling, in response to EGF

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Abstract

The adaptor protein Grb2 associates with phospholipase D2 (PLD2), but it is not known if this interaction is necessary for the lipase's functionality *in vivo*. In this study, we demonstrate that stable shRNA-based silencing of Grb2, a critical signal transducer of the epidermal growth factor receptor (EGFR) and linker to the Ras/Erk pathway, resulted in the reduction of PLD2 activity in COS7 cells. Transfection of a Grb2 construct refractory to shGrb2 silencing ($XGrb2^{SiL}$) into the Grb2-knockdown cells ($COS7^{shGrb2}$), resulted in the nearly full rescue of PLD2 activity. However, Grb2-R86K, an SH2-deficient mutant of Grb2 that is incapable of binding to PLD2, failed to induce an enhancement of the impaired PLD2 activity in $COS7^{shGrb2}$ cells. Grb2 and PLD2 are directly associated and Grb2 is brought down with anti-myc antibodies irrespectively of the presence or absence of EGFR activation. Immunofluorescence microscopy showed that co-transfected PLD2 and Grb2 re-localize to Golgi-like structures after EGF stimulation. Since this was not observed in cotransfection experiments of Grb2 and PLD2-Y169/179F, a lipase mutant that does not bind to Grb2, we inferred that Grb2 serves to hijack PLD2 to the perinuclear Golgi region through its SH2 domain. Supporting this is the finding that the primary cell line HUVEC expresses PLD2 diffusely in the cytoplasm and in the perinuclear Golgi region, where PLD2 and Grb2 colocalize. Such colocalization in primary cells increased after stimulation with EGF. In summary, these results demonstrate for the first time that the presence of Grb2 and its interaction with localized intracellular structures is essential for PLD2 activity and signaling *in vivo*.

Keywords

PLD2; Grb2; shRNA; co-localization; site-directed mutagenesis

INTRODUCTION

Two human phospholipase D (PLD) genes have been described: PLD1 and PLD2[1; 2], as well as their regulators[3]. The expressed proteins contain two invariable sequences, $HxK[x]_4D$ $[x]_6GSxN$, or HKD, that are responsible for enzymatic activity, as well as several conserved regions such as a PIP_2 binding site, a plekstrin-homology (PH) domain and a *phox* (PX) domain[4]. PLDs hydrolyze phosphatidylcholine to produce a lipidic second messenger, PA, and choline. We have recently demonstrated that residues Y^{169} and Y^{179} expressed within the

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context of the consensus YXNX in the PX domain of PLD2 directly bind the SH2 motif of Grb2[5], but the *in vivo* implications have remained elusive.

The central SH2 domain of Grb2 binds tyrosine phosphorylated residues within the consensus sequence YXNX[6; 7], whereas two flanking SH3 domains interact with proline-rich regions of other proteins, such as those found in the Ras guanine nucleotide exchange factor, Sos[8; 9; 10; 11]. The Grb2/Sos complex promotes GTP loading of Ras, which leads to the activation of Ras effectors. As such, it has multiple functions in embryogenesis, cancer, regulation of the cytoskeleton, cell differentiation and DNA synthesis[9; 12; 13; 14].

A well know effect of the Grb2/Sos complex is to engage the EGFR with cell proliferation *via* direct recruitment and activation of the MAPK signaling pathway[9]. Stimulation of COS7 cells with EGF causes the cytosolic Grb2 to partially relocate to the plasma membrane (the place where the interaction of EGFR to Grb2 occurs) which is followed by localization in vesicle-like structures at the perinuclear region of the Golgi apparatus[15; 16]. An intracellular relocalization of PLD2 followed EGF treatment has been explored before in mouse[17; 18; 19], but a connection between PLD2 and Grb2 has not been addressed experimentally as of yet.

Our laboratory has recently demonstrated that PLD2 interacts with the Grb2/Sos complex[5; 20], and here we sought to ascertain how critical the presence of Grb2 was for the functionality (activity and intracellular localization) of the lipase *in vivo*. For this, we generated a new COS7 cell line that continuously expressed a short hairpin RNA and exhibited silenced expression of Grb2. In this experimental model, Grb2 expression and function were rescued *via* transient transfection of *Xpress*-tagged Grb2 constructs which were made refractory to the action of the shRNA-mediated interference. This has enabled us to demonstrate for the first time an essential role of Grb2 in PLD2 activity and intracellular localization in response to growth factor stimulation *in vivo*.

RESULTS

Knocking-down and rescuing Grb2

COS7 cells deficient in endogenous Grb2 expression were generated by stably transfecting them with pU6^{+27}-shGrb2 or pU6^{+27}-shControl. After selection of stable cells, expression of endogenous Grb2 (~25 kDa) was analyzed by Western blot. As shown in Figures 1A and 1B, Grb2 expression was minimal (0–10 %) in COS7 cells stably expressing pU6^{+27}-shGrb2 (COS7^{shGrb2}, clones #1–4), whereas COS7^{shControl} and COS7^{WT} cells, both expressed comparable normal levels of the Grb2 protein, demonstrating that endogenous Grb2 can be knocked down in COS7 cells. Since silencing specificity is corroborated by the ability to rescue the shRNA-mediated silencing[21], we created a mutant version of the targeted gene that is not recognized by the shRNA. We introduced silent changes in the human Grb2 cDNA encoded in the construct pcDNA-XGrb2[5]. The new plasmid (named pcDNA-XGrb2^{SiL}) was transfected into COS7^{shGrb2} cells (clone #4), and after 48 hours, XGrb2^{SiL} protein expression was analyzed by Western blot using antibodies against human Grb2. As shown in Figure 1C, XGrb2^{SiL} (~30 kDa) is detected whereas endogenous Grb2 (~25 kDa) is minimal or undetectable in COS7^{shGrb2} cells transfected with pcDNA-XGrb2^{SiL} WT. Normal XGrb2^{SiL} WT expression and localization in COS7^{shGrb2} cells were demonstrated by direct immunofluorescence using a FITC-conjugated anti-*Xpress* antibody. As shown in Figure 1D, XGrb2^{SiL} transiently expressed in COS7^{shGrb2} cells (clone #4) localizes diffusely in the cytoplasm. This is in agreement to other authors' findings for different Grb2 constructs in COS^{WT} cells[16]. Taken together, these results demonstrate that endogenous Grb2 expression can be specifically knocked-down and rescued in COS7^{shGrb2} cells.

Functional Grb2 rescuing in COS^{shGrb2} cells

It is known that upon EGF stimulation of COS7^{WT} cells, a fraction of cytosolic Grb2 becomes redistributed to the plasma membrane and to endosome-like structures at the perinuclear Golgi region[16; 22]. Simultaneously, p42/44^{ERK} become phosphorylated in residues T^{202}/Y^{204} promoting their activation[9]. We sought to investigate whether those two phenomena would be present in our system at hand. For this, COS7^{shGrb2} cells, transiently expressing XGrb2^{SiL}, were stimulated with EGF and analyzed for immnofluorescence and Western blotting. As shown in Figure 1D and 2A (left), XGrb2^{SiL} is expressed diffusely in the cytoplasm of the COS7^{shGrb2} cells (clone #4) in the absence of EGF. However, after 3–5 min of EGF stimulation (100 ng/ml), it could be seen that XGrb2^{SiL} had re-localized to and concentrated in the perinuclear Golgi region of COS7^{shGrb2} cells (Figure 2A, right). Figure 2B shows that a robust detection of Grb2 could be observed in XGrb2^{SiL}-transfected COS7^{shGrb2} cells (clone #4). Concurrently, serum-starved COS7^{shControl} cells showed the expected increase in T^{202}/Y^{204} phosphorylation of p42/44^{ERK} upon EGF treatment whereas activation of p42/44^{ERK} is dramatically impaired in COS7^{shGrb2} cells (Figure 2C). However, EGF-induced phosphorylation of p42/44^{ERK} could be rescued in COS7^{shGrb2} transiently expressing XGrb2^{SiL} WT (Figure 2B, middle lanes). Taken together, these results demonstrate that Grb2 can be functionally rescued in COS7^{shGrb2} cells.

PLD2 activity is impaired in COS^{shGrb2} cells and rescued with XGrb2^{SiL}

Human PLD2 interacts with Grb2 *in vivo*[20] and the complex PLD2/Grb2 is catalytically active *in vitro*[20]. We tested the role of Grb2 on PLD2 activity in COS7^{shGrb2} and control cells (COS7^{WT} or COS7^{shControl}) transiently transfected with pcDNA-mycPLD2 WT or with the lipase inactive mutant K758R. Forty eight hours post-transfection, cell lysates were obtained and tested for total PLD2 activity. Figure 3A (*top*) shows that transient expression of mycPLD2 WT in COS7^{WT} or COS7^{shControl} cells results in an ~800 % increase in PLD2 activity when compared to mock- or mycPLD2 K758R-transfected cells. However, PLD2 activity was impaired (~80 %) in COS7^{shGrb2} cells. Further, PLD2 activity correlated with decreased levels of Grb2 protein expression when compared to COS7^{WT} or COS7^{shControl} cells (Figure 3A, *bottom*). Lastly, we wanted to investigate if the impaired PLD activity in COS7^{shGrb2} cells could be rescued by the XGrb2^{SiL} plasmid. Figure 3B shows that the reduced level of enzyme activity in PLD2-transfected COS7^{shGrb2} cells could be greatly recuperated in the XGrb2^{SiL} added-back experiments (Figure 3B, third bar). Most impressively, the rescue resulted in achieving about 75% of the original high level of PLD activity of COS7^{WT} cells (Figure 3A, outer bars). Conversely, pcDNA-XGrb2^{SiL} R86K, an SH2-deficient version of Grb2 could not do the same (Figure 3B, top and bottom panels). These results suggest that normal cellular levels of fully functional Grb2 are required for PLD2 in order to be fully active.

PLD2 Y169/179F cannot bind the Grb2/Sos complex or activate Ras and is catalytically inactive

Mutation or deletion of PLD2 residue Y^{179} releases Y^{169} functionality[5]. Indeed, residues Y^{169}/Y^{179} in PLD2 are necessary to bind Grb2, whereas only the complex PLD2-Y^{169}/Grb2 (observed in PLD2 Y179F) activates Ras[5]. Hence, it is tempting to speculate that PLD2 Y169/179F is a catalytically incompetent enzyme unable to activate Ras. In order to confirm this hypothesis, COS7^{WT} cells were first transiently transfected with pcDNA-mycPLD2 WT or Y169/179F plasmids and after 48 hours, *in vivo* PLD2 interaction with the Grb2/Sos complex analyzed by co-immunoprecipitation. As shown in Figure 4A, PLD2 WT interacts with Grb2/Sos, as demonstrated previously[5; 20]. However, PLD2 Y169/179F mutant was unable to co-immunoprecipitate the Grb2/Sos complex. Moreover, PLD2 Y169/179F was unable to activate Ras or p42/44^{ERK}, suggesting that Grb2 binding to PLD2 plays an essential role in MAPK activation (Figure 4B). Simultaneously, COS7^{WT} lysates transiently expressing mycPLD2

WT, Y169/179F or K758R were analyzed for total enzymatic activity. As shown in Figure 4C, PLD2 Y169/179F is catalytically inactive, suggesting that PLD2 association to Grb2 is involved in high basal PLD2 activity. Moreover, Grb2 association to PLD2 appear to be independent of EGFR stimulation of COS7^{shGrb2} cells (Figure 5).

Grb2 and PLD2 colocalize in the perinuclear Golgi region upon cell stimulation

Since Grb2 interacts with PLD2, we tested the possibility that PLD2 follows Grb2 localization upon stimulation of COS7^{shGrb2} with EGF. To this end, COS7^{shGrb2} cells were transiently transfected with pcDNA-mycPLD2 WT and/or pcDNA-XGrb2^{SiL} WT for 48 hours. After this time, cells were exposed to vehicle or 100 ng/ml EGF for 5 min and analyzed for mycPLD2 and/or XGrb2^{SiL} immunolocalization. We observed that regardless of basal or EGF-stimulated conditions, COS7^{shGrb2} cells (lacking endogenous Grb2 protein) and transfected with pcDNA-mycPLD2 WT onlone express most of the mycPLD2 throughout the cell in the cytoplasm and to a lesser extent in vesicular structures in the perinuclear Golgi region (Figure 6A). However, when pcDNA-mycPLD2 WT was co-expressed with pcDNA-XGrb2^{SiL} WT, a dramatic redistribution of mycPLD2 from the cytoplasm to the perinuclear Golgi region of COS7^{shGrb2} cells occurred, an effect that was clearly observed after 5 min stimulation with EGF (Figure 6B, *top panel*). Moreover, mycPLD2 appears to co-localize with XGrb2^{SiL} in the cytoplasm and perinuclear Golgi region of unstimulated COS7^{shGrb2} cells whereas mycPLD2 follows XGrb2^{SiL} relocalization to the perinuclear Golgi region upon EGF treatment (Figure 6B, *center and botom panels*). Since mycPLD2 staining was almost lost in the cytoplasm of COS7^{shGrb2} cells co-expressing XGrb2^{SiL}, these results suggest that XGrb2^{SiL} hijacks mycPLD2 in the perinuclear Golgi region, an effect more dramatically seen upon EGF stimulation.

Reinforcing this was the observation that mycPLD2-Y169/179F, an inactive mutant incapable of binding Grb2 or activate Ras *in vivo* (Figure 4), could not cause an intracelluar redistribution of Grb2 in response to EGF as mycPLD2 WT did in COS7^{shGrb2} cells co-expressing XGrb2^{SiL} (Figure 7). Hence, these results indicate that mycPLD2 localization in the preinuclear Golgi region in EGF-stimulated cells depends on Grb2, probably *via* SH2-mediated interaction of Grb2 with residues Y^{169}/Y^{179} in PLD2.

Endogenous Grb2 and PLD2 colocalize in the perinuclear Golgi region of primary cells

Although a fraction of Grb2 redistributes to the plasma membrane and perinuclear region upon EGF stimulation, PLD2 does not appear to follow Grb2 to the membrane, but to the Golgi region. We test the possibility that endogenous expression of Grb2 and PLD2 could redistribute in primary cells in a way similar to that observed in transfected cells upon EGF stimulation. Regardless EGF stimulation, primary HUVEC cells express endogenous PLD2 in the cytoplasm and the perinuclear Golgi region (Figure 8A). On the other hand, under basal conditions, HUVEC cells express Grb2 finelly dispersed in the cytoplasm whereas upon EGF stimulation, a portion of Grb2 is redistributed to the plasma membrane and the perinuclear Golgi region (Figure 8B), as demonstrated in several cell types [23]. Moreover, PLD2 colocalizes with Grb2 in the prinuclear region, and this colocalization becomes more pronounced upon EGF stimulation (Figure 8C). Taken together, these results suggest that PLD2 follows Grb2 localization to especific compartments, but not to the plasma membrane upon EGF stimulation.

DISCUSSION

We have used RNA interference to generate a COS7 cell line expressing less than 10 % of normal levels of Grb2 and to demonstrate the vital role of Grb2 in PLD2 physiology. Silencing endogenous Grb2 in COS7 cells is specific since COS7^{shGrb2} cells expressed pcDNA-XGrb2^{SiL}, an *Xpress*-tagged Grb2 version made silent in order to by-pass the shGrb2 actions,

is expressed in COS7^{shGrb2} cells (Fig. 1). Transient expression of XGrb2^{SiL} did not result in acute upregulation of endogenous Grb2 in COS7^{shGrb2} cells due to transfection artifacts, demonstrating that endogenous Grb2 silencing can be rescued at the protein level. Moreover, critical functions of Grb2 in response to EGF (*i.e.* Grb2 redistribution and p42/44^{ERK} activation) were rescued when COS7^{shGrb2} cells expressed XGrb2^{SiL} (Fig. 2). These results suggest that stable shRNA-mediated Grb2 silencing, as well as its consequences, in COS7^{shGrb2} cells can be effectively rescued by transient expression of XGrb2^{SiL}.

Grb2 interacts with PLD2[20] *in vivo* and the association Grb2/PLD2 occurs *via* specific interaction of the SH2 domain of Grb2 with residues Y^{169}/Y^{179} in PLD2[5]. In the present work, we demonstrate that permanent silencing of endogenous Grb2 correlates with impaired PLD2 activity (Fig. 3A). Residual levels of PLD2 activity could be demonstrated in COS7^{shGrb2} expressing mycPLD2 WT, probably reflecting the fact that residual expression of Grb2 (<10 %) is invariably present in COS7^{shGrb2} cells. Nevertheless, impaired PLD2 activity in COS7^{shGrb2} cells could be rescued by transient expression of XGrb2^{SiL} WT demonstrating the specific role of Grb2 in PLD2 high basal activity (Fig. 3B).

Although the mechanisms involved in Grb2-mediated regulation of PLD2 activity are still unknown, when mycPLD2 was cotransfected with the SH2-deficient mutant XGrb2^{SiL} R86K, PLD2 activity was not restored indicating that mycPLD2 activity requires the SH2 functionality of Grb2. These results recapitulate the proposed phenomena in which the SH2 domain of Grb2 interacts with PLD2 residues Y^{169}/Y^{179}, whilst the SH3 domains of Grb2 are involved in PLD2 activity[5]. Moreover, we extend our previous results by demonstrating that PLD2 Y169/179F is a catalytically inactive enzyme that cannot either interact with the Grb2/Sos complex or activate Ras in COS7^{WT} cells (Fig. 4). Taken together, our results confirm the role of functional Grb2 as a critical regulator of PLD2 activity in COS7 cells.

The components and functionality of Ras signaling, including Grb2 and Sos, have been localized on endosomes and the perinuclear Golgi region[24; 25]. Under basal conditions, Grb2 is an entirely cytoplasmic protein[15; 16]. However, upon EGF stimulation of COS7^{WT} cells, a fraction of the cytosolic Grb2 is redistributed to the plasma membrane and/or large vesicle-like structures, where its SH2 domain interacts directly with the EGFR[15]. After 5 minutes of EGF stimulation, EGFR, Grb2 and Ras are concentrated in large macropinocytic structures and in perinuclear endosome-like vesicles[16; 22; 23].

The role of PLD2 in receptor-mediated endocytosis is established [18; 26]. Interestingly, residues R^{128}/R^{197} in the PX domain of PLD1, or R^{110}/R^{180} in PLD2, are essential for EGF-mediated EGFR endocytosis by acting as a GAP for dynamin, known to pinch off vesicles from the membranes[27]. As demonstrated recently, R^{180} is part of the PLD2 consensus for the interaction with the SH2 domain of Grb2[5]. *Early reports have shown that murine PLD2 localizes in the plasma membrane of different cells* [17; 26; 28]. The EGFR associates with the Y^{11} of the mouse PLD2 in HEK293 cells[17]. However, Y^{11} is not conserved in human PLD2, suggesting that the known physical and functional interactions of murine PLD2 with the EGFR may be either species-specific and/or may occur indirectly, as sugegsted [27].

Regardless of basal or EGF-stimulated conditions, COS7^{shGrb2} cells express most of the PLD2 enzyme throughout the cell in undefined structures of the cytoplasm and to a lesser extent in vesicular structures in the perinuclear Golgi region (Fig. 6A). This localization is the opposite to what is observed by Freyberg *et al.* in rat GH3 cells[29] and by us in COS7^{WT} or COS7^{shControl} cells (*data not shown*). In these cells, most of the endogenous PLD2 was localized to the perinuclear Golgi region, whereas a small fraction of PLD2 was observed to be finely dispersed in the cytoplasm. Since the only difference between the present study and

that of Freyberg's is that our cells do not have Grb2, the logical conclusion is that the intracellular localization of PLD2 may be dependent on Grb2.

In line with this reasoning, COS7^{shGrb2} cells coexpressing mycPLD2 WT and XGrb2^{SiL} showed PLD2 redistribution in the perinuclear Golgi region and in to a lesser extent finely dispersed in the cytoplasm of the cell (Fig. 6B, *top panel*), as demonstrated by others in Grb2-containing cells[29]. Interestingly, under basal conditions, XGrb2^{SiL} and mycPLD2 WT colocalized in vesicle-like structures throughout the cytoplasm of the cell, but almost exclusively in the Golgi region after EGF treatment (Fig. 6B, *center and bottom panels*). These results suggest that mycPLD2 localization to the Golgi requires Grb2, not *viceversa* since Grb2 could relocalize to the perinulcear Golgi region in the absence of PLD2 coexpression (Fig. 2A). In agreement with these conclusions are the findings that XGrb2^{SiL} coexpression could not effectively rescue mycPLD2 Y169/179F localization, as with mycPLD2 WT. Indeed, a large portion of PLD2 Y169/179F remained in the cytoplasm of COS7^{shGrb2} cells, regardless EGF treatment (Fig. 7, *top panel*) and/or XGrb2^{SiL} coexpression (Fig. 6 *center and bottom panels*). Interestingly, upon 3–5 min of EGF stimulation, XGrb2^{SiL} could be detected in the plasma membrane, large cytoplasmic vesicles and in the perinuclear Golgi region, suggesting normal response of Grb2 to EGF in COS7^{shGrb2}, whereas a fraction of PLD2 Y169/179F and XGrb2^{SiL} still colocalized around the nucleus of the cell in undefined structures, suggesting that Grb2 cannot completely drag PLD2 Y169/179F into the perinuclear region in response to EGF. Taken together, these results suggest that the PLD2/Grb2 complex formation is necessary for PLD2 to efficiently localize in the perinuclear Golgi region upon EGF stimulation.

EGF causes relocalization of an important fraction of cytoplasmic Grb2 to the perinuclear Golgi region and endocytic vesicles [23], and also to the Golgi and endoplasmid reticulum [25]. Our results suggest that PLD2 localizes mainly in the perinuclear Golgi region and a smaller portion difusely in the cytoplasm, as demonstrated [29]. However, we privide new evidence suggestion that the normal perinuclear Golgi region distribution of PLD2 is dependent on Grb2 expression, since its depletion from COS7 cells causes that PLD2 is redistributed mainly to the cytoplasms in a fine distribution, just the opposite as previously demonstrated [29]. Interestingly, we observed that PLD2 follows Grb2 localization is more pronounced when cells are treated with EGF. It is known that EGF (1 min or less) causes a portion of Grb2 to immediately translocate to the membrane and then Grb2 travels in endomsome-like vesicles and accumulates in the perinuclear Golgi region (after 5 min EGF treatment [23; 25]. However, when PLD2 and Grb2 are coexpressed in COS7 depleted of endogenous Grb2, most of the PLD2 signal goes to the perinuclear Golgi region and this is more evident after EGF treatment. Thus, these observation support the notion that Grb2 retains PLD2 in the perinuclear Golgi region and that this phenomena is more noticeable after 5 min treatment with EGF. In line, this Grb2-dependent "hijaking' of PLD2 is not observed when PLD2 Y169/179F is used, suggesting that the SH2 domain of Grb2 is involved in the retention of PLD2 in the perinuclear Golgi region.

In conclusion, we found that stable silencing of endogenous Grb2 correlates with impaired PLD2 activity and intracellular localization. This phenomena depends on the especific interaction of Grb2 with PLD2. This precise level of knowledge of PLD2 signaling at the molecular level will undoubtedly help our research of the key roles this enzyme plays in cell survival, growth, differentiation and oncogenesis.

MATERIAL & METHODS

Antibodies and Materials

The anti-Grb2 monoclonal antibody and the Ras Activation Assay Kit were from Upstate (Lake Placid, NY). The myc-tag, p42/44^{ERK}, pp42/44^{ERK}, Sos or EGFR antibodies were from Cell Signaling Technology (Boston, MA). DMEM, Lipofectamine and Plus reagents, Opti-MEM,

DNase I and anti-Xpress antibodies were from Invitrogen (Carlsbad, CA); electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA). Precast 4–20% polyacrylamide gels were from Pierce (Rockford, IL). Human recombinant EGF was from PreproTech, Inc. (Rocky Hill, NJ).

Plasmids

The shRNA Grb2 encoding construct used for Grb2 silencing (pU6^{+27}-shGrb2) and its control (empty vector, pU6^{+27}-shControl) were from Panomics, Inc (Fremont, CA). The human U6 snRNA promoter, the first 27 bp of the U6 snRNA (U6^{+27}) and the shRNA against human Grb2 were confirmed by direct sequencing (*data not shown*). Site-directed mutagenesis was used to generate silent versions of *Xpress*-tagged Grb2 (XGrb2) encoded in the construct pcDNA-XGrb2[5] and renamed pcDNA-XGrb2^{SiL}.

Mutagenesis of pcDNA-XGrb2^{SiL}

The construct pcDNA-XGrb2 plasmid was described in detail elsewhere[5]. pcDNA-XGrb2^{SiL} was generated by introducing seven silent mutations without changing the amino acid sequence of human Grb2 to make XGrb2^{SiL} resistant to the continuous presence of Grb2 shRNA (*nt* #310–330: GAT GTG CAG CAC TTC AAG GTT). The plasmid pcDNA-XGrb2 was used as a template for site directed mutagenesis following the QuickChange XL site-directed mutagenesis protocol (Stratagene, La Jolla, CA). The sense primer for the mutagenesis was: 5'-^{293}CT GTC AAG TTT GGA AAC GA**C** GT**C** CA**A** CA**T** TT**T** AA**A** GT**A** CTC CGA GAT GGA GCC GGG^{348}-3' (in bold are silently-mutated). The SH2-deficient version of pcDNA-XGrb2^{SiL} was generated by point-mutating the critical R^{86} residue within the SH2 domain of Grb2 (R86K). The mutant inserts were PCR-amplified and each point mutation checked by restriction analysis and full DNA sequencing.

Generation of a stable COS7^{shGrb2} cell line

COS7 were initially seeded at 1x10^5 cells/well in 6-well tissue culture plate in 1.5 ml D-MEM containing 10 % FBS. Cells were grown at 37°C in a CO_2 incubator until the cells were ~70% confluent. Stable transfection of COS7 cells with pU6^{+27} plasmids was done with 3 μg of linearized vectors. Two days after, cells were incubated in the presence of neomycin (0.5 μg/μl). Once all untransfected COS7 cells were dead in the presence of neomycin, COS7 cells resistant to neomycin were isolated and tested for pU6^{+27} plasmid integration into genomic DNA by PCR (*data not shown*). Double-positive clones were diluted 1:2000 and plated in the presence of neomycin. After ~2 weeks, single colonies were selected and and tested for endogenous expression if Grb2 by Western blot. Four COS7 clones expressing minimal endogenous Grb2 (0–10 %, named: COS7^{shGrb2}) and four control clones expressing normal levels of Grb2 (named: COS7^{shControl}) were selected, propagated and used for transient transfection with constructs expressing PLD2 (pcDNA-mycPLD2) and/or silent versions of human Grb2 (pcDNA-XGrb2^{SiL}) *In vitro PLD activity.* Phospholipase D activity (transphosphatidylation) in cell extracts was measured in liposomes of short-chain PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (PC8), and ^{3}H-butanol s described in detail elsewhere[30].

Western blotting, Erk and Ras activation

Twenty μg total COS7 protein were subjected to SDS-PAGE (4–20%), transferred to PDVF membranes, blocked and blotted following standard procedures. Erk activation was analyzed by immuno-detecting the phosphorylation state of p42/44^{ERK} at residues T^{202}/Y^{204} using specific antibodies that detected a doublet of phosphoproteins on Western Blots corresponding to *p*p42 and *p*p44 MAPKs. Ras activity was determined by using the Ras Activation Assay Kit following the instructions of the manufacturer. GTP-bound Ras was affinity-purified from 500

μg of COS7 lysates transiently expressing mycPLD2 WT or Y^{169}/Y^{179} mutants by using the Ras-binding domain (RBD) of Raf1 coupled to agarose beads (RBD-Raf1-Agarose, Upstate, NY). Samples were incubated with 7.5 μg RBD-Raf1-Agarose 1 h at 4°C with gentle rocking and the beads pelleted, washed and resuspended in 25 □l 2X Laemmli buffer. Before PAGE (4–20 %), samples were boiled 5 minutes in the presence of 50 mM dithiothreitol. Membranes were blotted by using anti-Ras antibody (Cell Signaling, MA) and Western blots developed by the ECL method (Amersham, UK).

Immunofluorescence

Forty-eight hours post-transfection $COS7^{shGrb2}$ cells were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100 at room temperature. Cells were incubated 4 hr at 4 ° C in PBS/10% FBS/0.1% Triton X-100 (PBS-T). For single transfections, cells were probed overnight at 4 °C using either the anti-Xpress, FITC-conjugated, or the anti-myc, TRITC-conjugated antibodies. For co-transfected cells, first immunoincubation was done overnight at 4 °C with anti-Xpress FITC-conjugated antibody and then with anti-myc TRITC. Cells were washed with PBS and incubated in a 1:2000 dilution of DAPI in PBS for 5 min to visualize nuclei staining. Each coverslip was mounted onto a glass slide using one drop of Vectashield (Vector Labs) mounting media per slide. Slides were kept in the dark until needed and were viewed using a Nikon Upright Eclipse 50i Tissue Culture Microscope, a Plan Fluor 100X/1.30 Oil objective and FITC, TRITC or DAPI fluorescence filters. Photomicrographs were obtained using a Diagnostics Instrument Spot 6 digital camera and MetaVue software.

Statistical analysis

The analysis of multiple intergroup differences in each experiment was conducted by one-way analysis of variance followed by Student test. A $p<0.05$ was used as the criterion for statistical significance.

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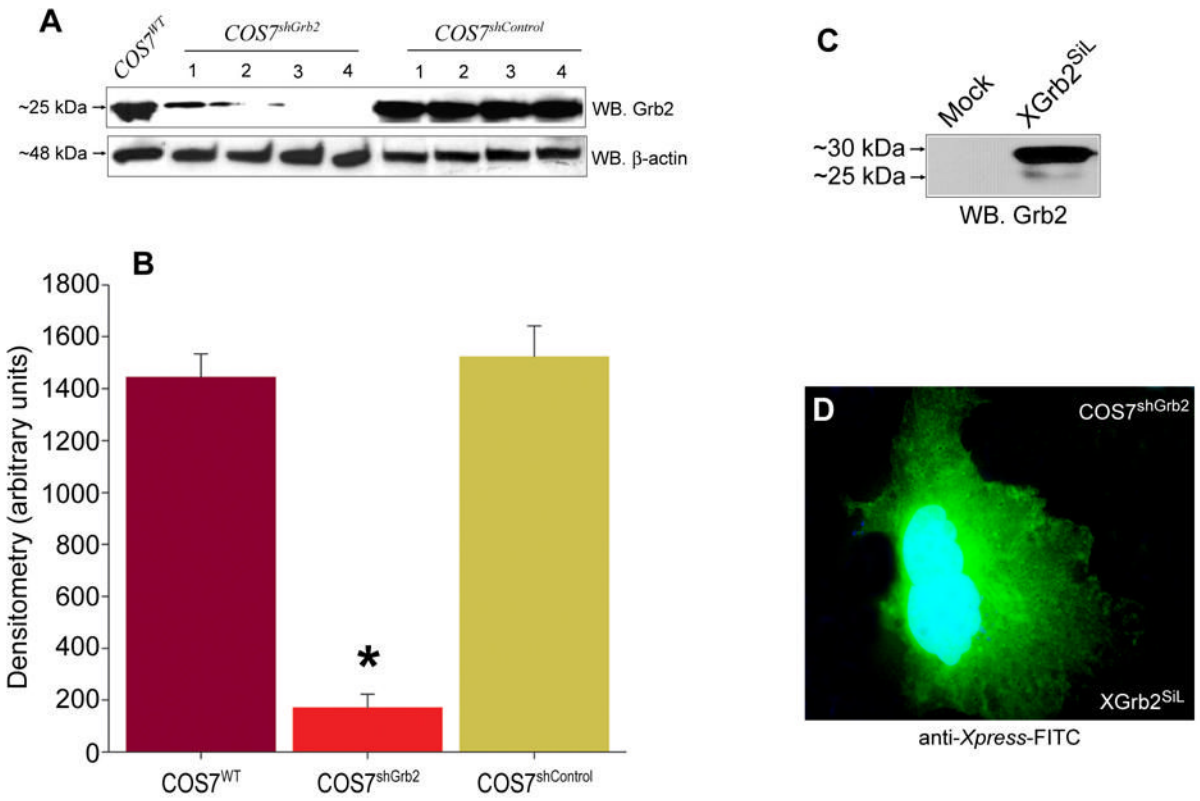

Figure 1. Expression of Grb2 in COS7^{shGrb2} cells

(A) Western blot estimation of endogenous Grb2 expression (~25 kDa) in COS7 cells wild-type (COS7^{WT}) and four different clones of stable transfected cells (COS7^{shGrb2} or COS7^{shControl}). As a loading Control, a b-actin blot of the transferred membrane is also shown. **(B)** Densitometric analysis of endogenous Grb2 expression in COS7^{WT}, COS7^{shGrb2} and COS7^{shControl} cells expressed as mean ± SEM. At least 5 different immunoblots were analyzed (*p<0.001). **(C)** Silenced expression of endogenous Grb2 in COS7^{shGrb2} cells is rescued by transient transfection of a "silent" version of Grb2 (XGrb2^{SiL}). COS7^{shGrb2} cells were transfected with 2 mg of pcDNA-XGrb2^{SiL} WT and the expression of endogenous Grb2 (~25 kDa) and transfected XGrb2^{SiL} (~30 kDa) were analyzed by Western blot using an anti-Grb2 antibody. Shown is a representative experiment. **(D)** Direct immunofluorescent detection of XGrb2^{SiL} WT in COS7^{shGrb2} cells. COS7^{shGrb2} cells transiently expressing pcDNA-XGrb2^{SiL} for 48 hours were serum-starved overnight and analyzed for XGrb2^{SiL} localization by direct immunofluorescence using a FITC-conjugated antibody against the *Xpress*-tag of XGrb2^{SiL}. Nucleus is DAPI-stained.

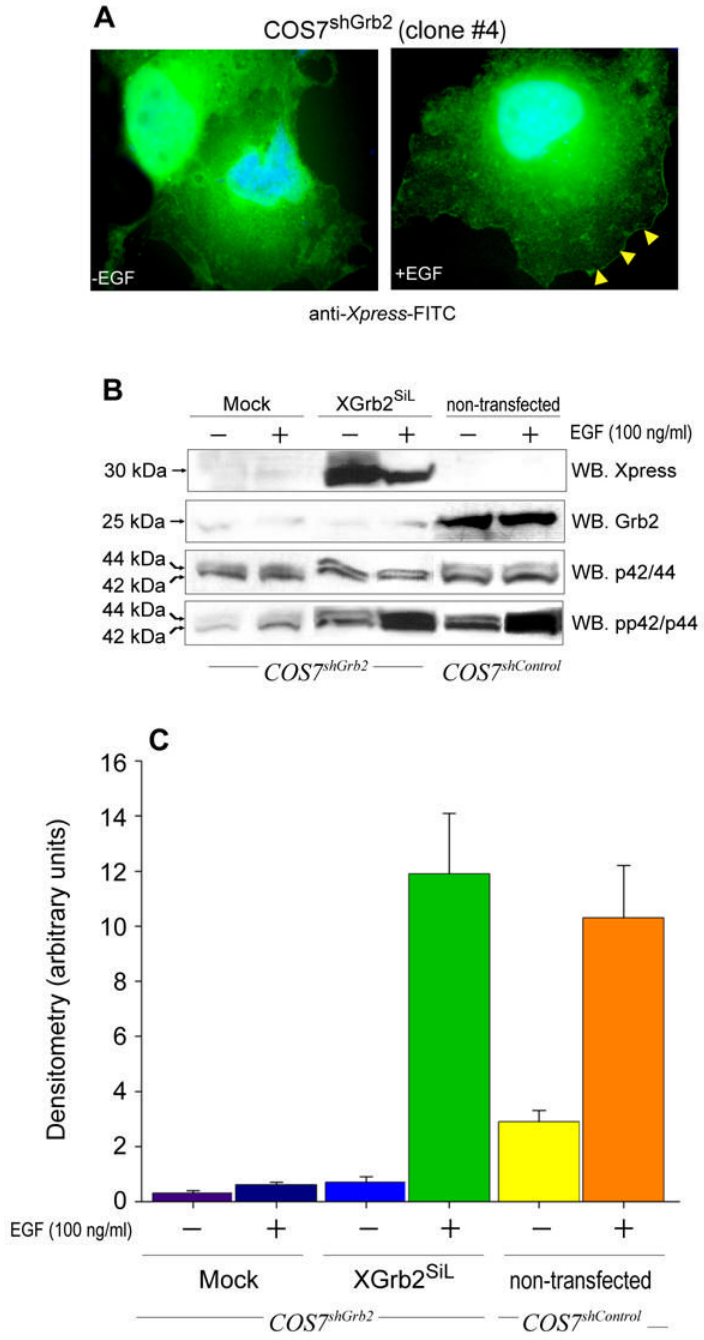

Figure 2. Rescuing Grb2 functionality in COS7^{shGrb2} cells

(A) COS7^{shGrb2} cells mock-transfected or transiently expressing pcDNA-XGrb2^{SiL} were serum starved overnight and treated 5 minutes with 100 ng/ml EGF. Immunolocalization of XGrb2^{SiL} was analyzed directly by using a FITC-conjugated anti-*Xpress* antibody. *Left*, Grb2 disperse cytoplasmic localization. *Right*, EGF-induced Grb2 redistribution to the plasma membrane (yellow arrowheads) and the perinuclear Golgi region. **(B)** Non-transfected COS7^{WT} or COS7^{shControl} and mock- or pcDNA-XGrb2^{SiL}-transfeted COS7^{shGrb2} cells were incubated for 30 hs. After this time, cells were serum-starved and treated with 5 min with EGF (100 ng/ml). Expression of XGrb2^{SiL}, Grb2 and phosphorylated (activated) p42/44^{ERK} were simultaneously analyzed by Western Blot using especific antibodies. Shown is a representative

experiment. **(C)** Densitometric analysis of phosphorylated $p42/44^{ERK}$ normalized to total $p42/44^{ERK}$ signal.

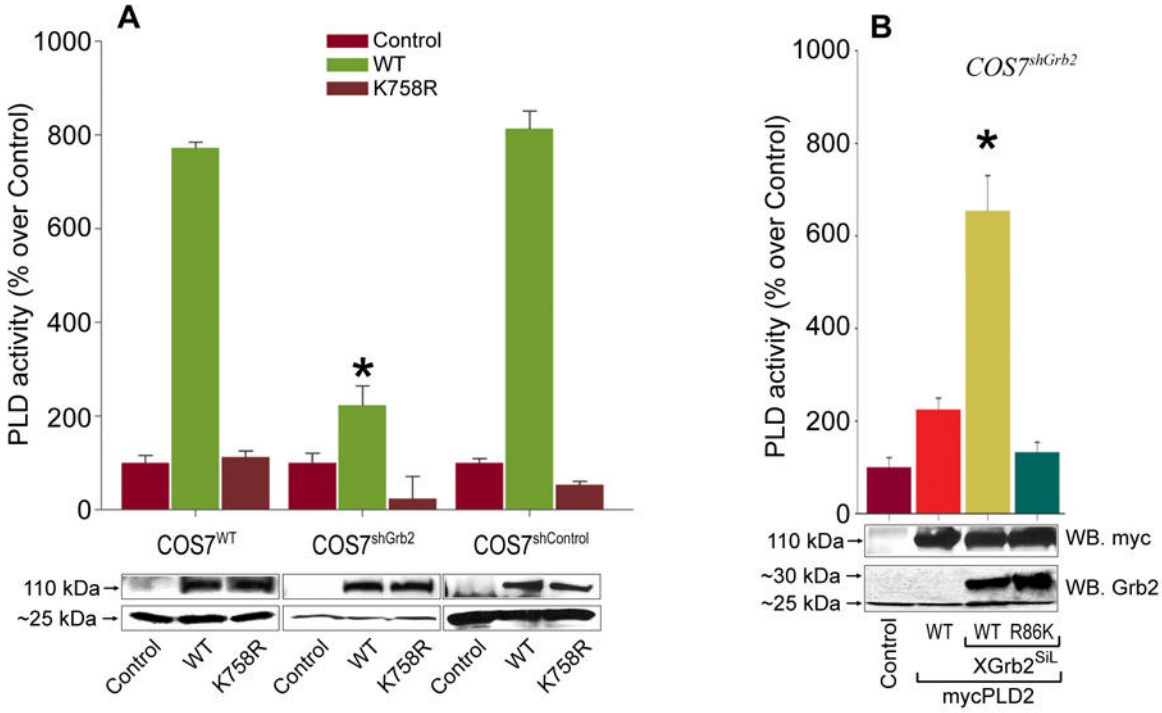

Figure 3. PLD2 catalysis is impaired in COS7^{shGrb2} but rescued by coexpressing XGrb2^{SiL}

(A, *top*) PLD2 activity in COS7^{shControl}, COS7^{shGrb2} or COS7^{WT} cells expressing pcDNA-mycPLD2 WT or K758R. COS7 cells were transiently transfected with 2 ug of pcDNA-mycPLD2 WT or the inactive mutant of PLD2, K758R. Thirty hours post-transfection, cellular lysates were obtained and PLD2 activity analyzed as the capacity of cell extracts to transphosphatidylate PC8 and [^{3}H]-butanol. Results are expressed as % PLD activity (dpm/well) over each control, as mean ± SEM of at least 5 experiments done in duplicate (* $p<0.001$). ***(bottom)*** PLD2 WT, K758R and endogenous Grb2 expression levels determined by myc or Grb2-immunoblot of whole COS7 cell lysates. **(B, *top*)** COS7^{shGrb2} cells were co-transfected with pcDNA-mycPLD2 WT and pcDNA-XGrb2^{SiL} (WT or R86K) vectors and total PLD2 activity analyzed after 48 hours. Results are expressed as % PLD2 activity (dpm/well) over each Control, as mean ± SEM of at least 3 different experiments done in duplicate (***** $p<0.001$). (**B, *bottom***) Expression of mycPLD2 WT, total Grb2 (~25 kDa) and XGrb2^{SiL} WT or R86K (~30 kDa) in whole lysates of co-transfected COS7^{shGrb2}. The same cellular whole lysates subjected to the PLD assay were analyzed for the expression of Grb2, XGrb2 and mycPLD2 by Western blot.

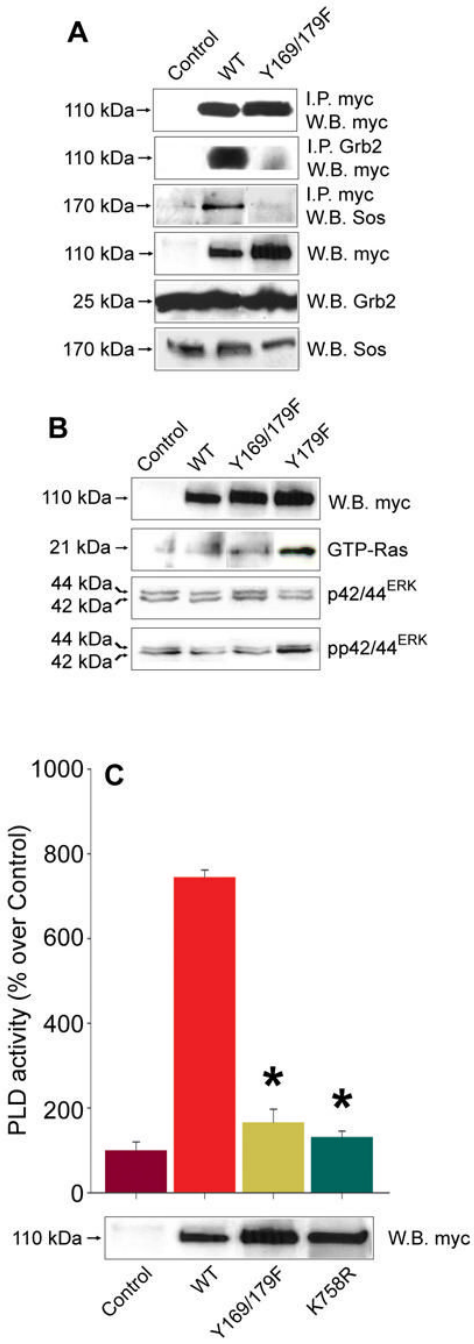

Figure 4. Human PLD2 Y169/179F is a catalytically inert enzyme which cannot interact with the Grb2/Sos complex or activate Ras

(A) Co-immunoprecipitation of mycPLD2 and/or Grb2 from COS7^{WT} cells transiently expressing pcDNA-mycPLD2 WT or Y169/179F. COS7^{WT} cells were transiently transfected with the indicated plasmids and after 48 hours, association of mycPLD2 with the Grb2/Sos complex analyzed by co-immunoprecipitation using myc or Grb2 antibodies. The immunological presence of mycPLD2, Grb2 and Sos in myc-immunoprecipitates are shown together with the expression of each protein in whole COS7^{WT} lysates. **(B)** Activation of the Ras/MAPK signaling pathway was analyzed by direct determination of active Ras (immunoipurified GTP-bound Ras) and by the degree of p42/44^{ERK} phosphorylation in

residues T/Y using immunoblot. Ras/MAPK activation was analyzed in whole lysates of $COS7^{WT}$ cells transiently expressing pcDNA-mycPLD2 WT, Y169/179F or Y179F (positive Control) for 48 hours. **(C, *top*)** Total PLD2 activity in $COS7^{WT}$ expressing mycPLD2 WT, Y169/179F or the inactive mutant K758R. Results are expressed in terms of % PLD activity (dpm/well) over Control, as mean ± SEM of at least 3 different experiments performed in duplicate (***** $p<0.001$). **(C, *bottom*)** Expression levels of each mycPLD2 variant in whole $COS7^{WT}$ lysates. The same samples analyzed for PLD2 activity were subjected to SDS-PAGE, transferred and blotted with anti-myc antibodies.

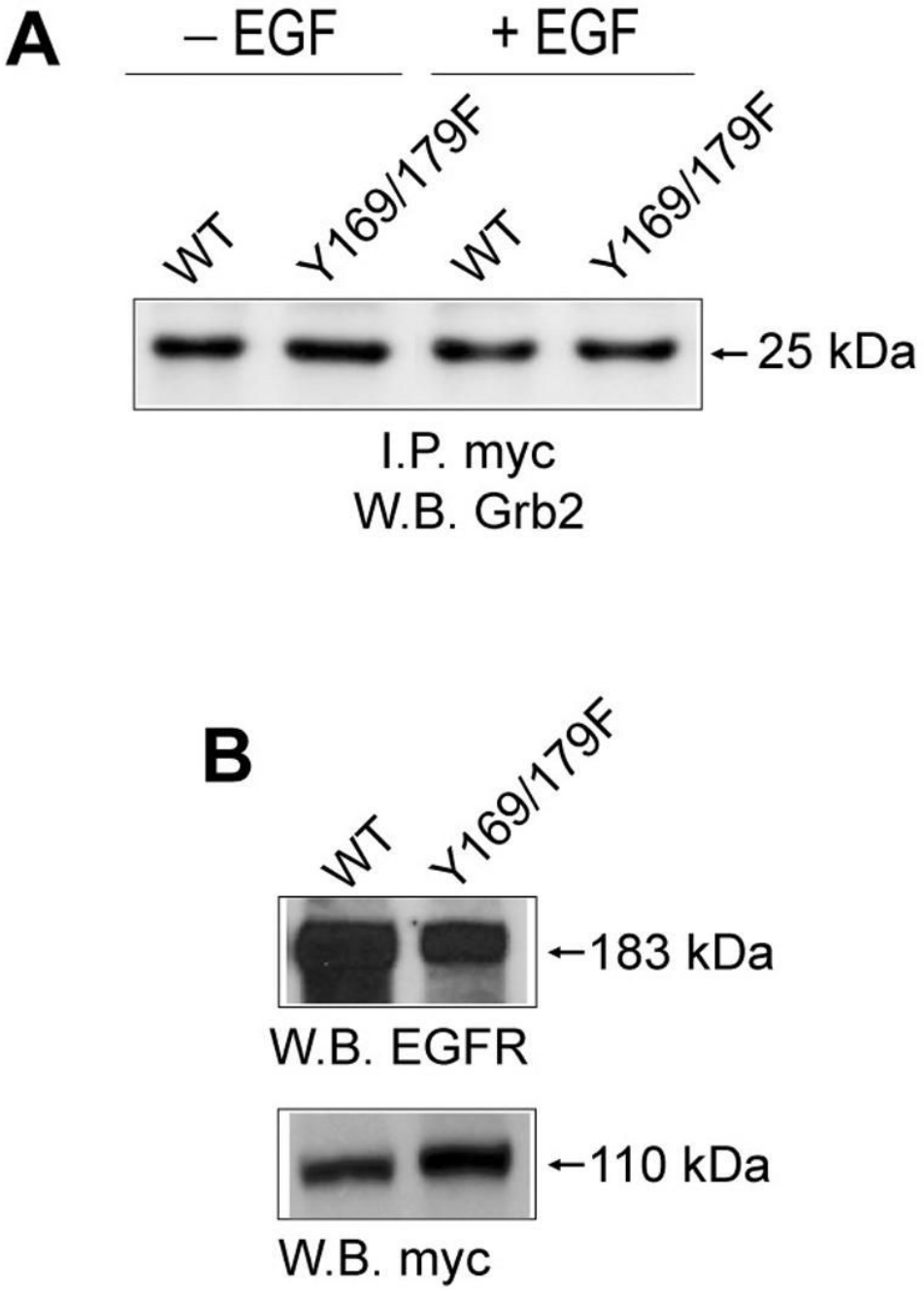

Figure 5. PLD2 co-immunoprecipitates with Grb2 regardless EGFR stimulation

(A). COS7^{shGrb2} cells transiently expressing mycPLD2 WT or Y169/Y179F were treated with EGF for 5 min. Cell lysates were obtained and immunoprecipitated with anti-myc antibodies. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-myc Grb2. **(B).** Shown are anti-myc and anti-EGFR immunoblots of whole COS7^{shGrb2} lysates overexpressing mycPLD2 WT or Y169/179.

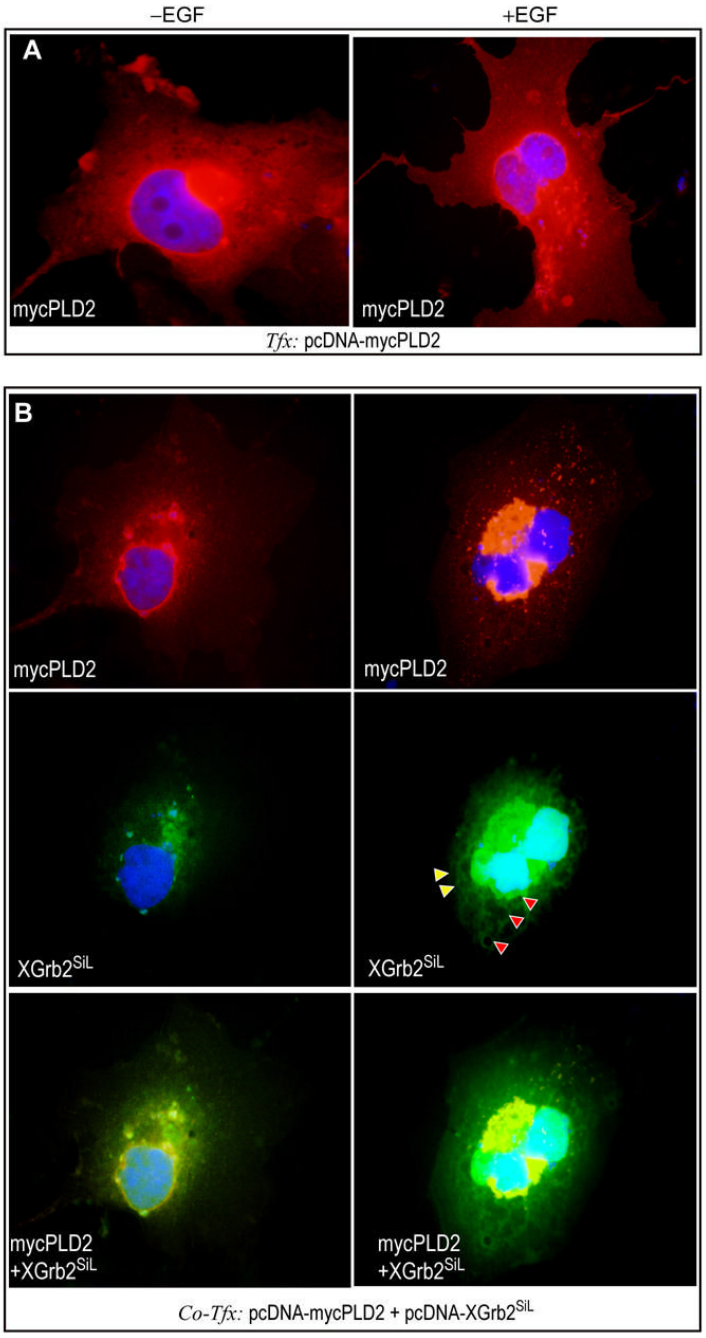

Figure 6. PLD2 colocalizes with Grb2 in the perinuclear Golgi region of COS7^{shGrb2} cells

(A) COS7^{shGrb2} cells were transiently transfected with pcDNA-mycPLD2 and after 48 hours cells were serum-starved overnight, incubated with or without 100 ng/ml EGF for 5 minutes and analyzed for mycPLD2 localization by direct immunofluorescence using a TRITC-conjugated anti-myc antibody. Shown are representative pictures of mycPLD2 distribution in COS7^{shGrb2} treated or not with EGF. **(B)** COS7^{shGrb2} cells were transiently cotransfected with pcDNA-mycPLD2 and pcDNA-XGrb2^{SiL} for 48 hours. Cells were serum-starved overnight, incubated with or without 100 ng/ml EGF for 5 minutes and analyzed for mycPLD2 localization (*upper panel*), XGrb2^{SiL} localization (*central panel*) or both (*lower panel*), by direct immunofluorescence using a TRITC- anti-myc or FITC-conjugated anti-Xpress antibodies.

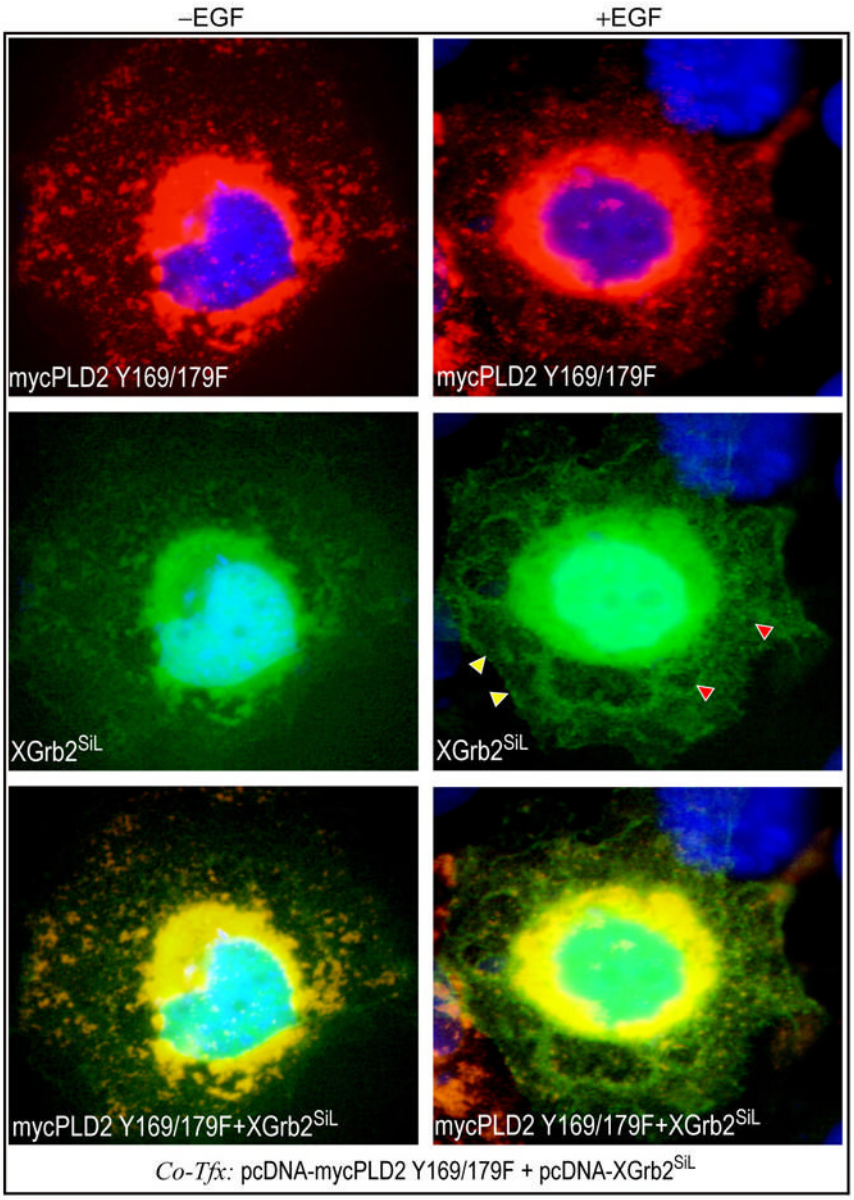

Figure 7. PLD2 Y169/179F cannot colocalize with Grb2

COS7^{shGrb2} cells transiently co-expressing pcDNA-mycPLD2 Y169/179F and pcDNA-XGrb2^{SiL} for 48 hours were serum-starved overnight. After this, cells were incubated with or without 100 ng/ml EGF for 5 minutes, fixed, immunolabeled and analyzed for mycPLD2 Y169/179F localization (*upper panel*), XGrb2^{SiL} localization (*central panel*) or both (*lower panel*), by direct immunofluorescence using a TRITC- anti-myc or FITC-conjugated anti-Xpress antibodies.

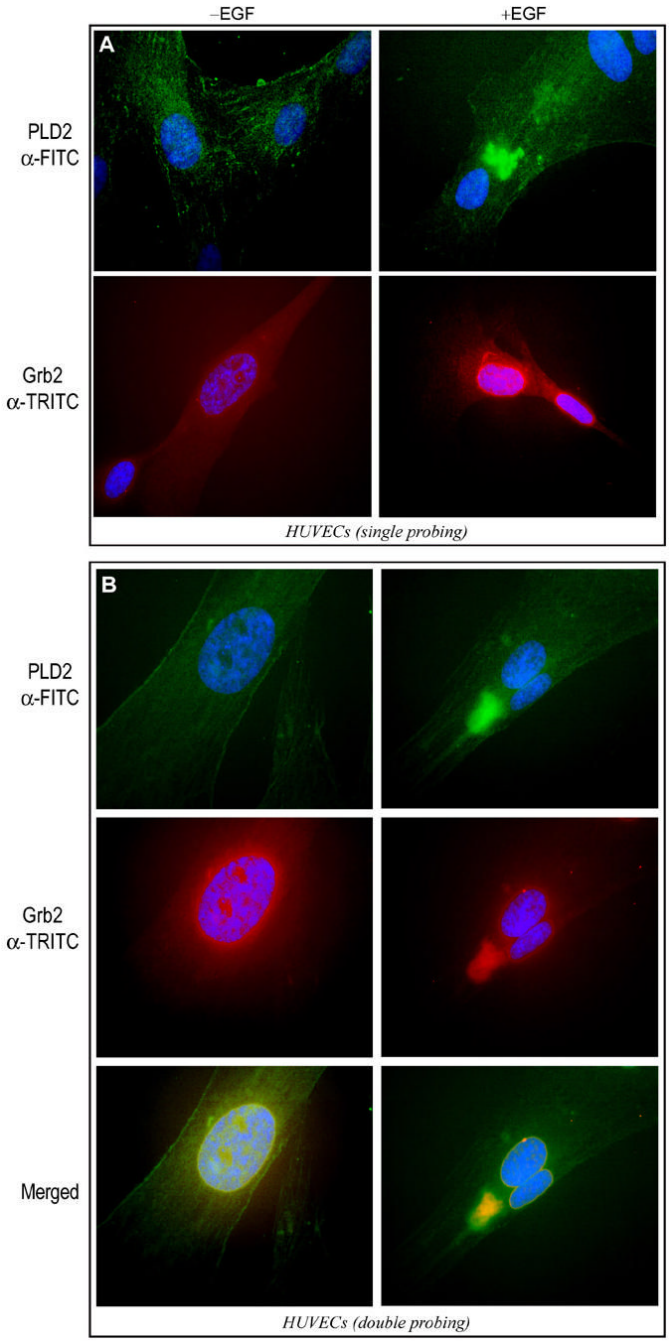

Figure 8. Expression and localization of endogenous PLD2 and Grb2 in HUVEC primary cells

The endogenous expression and cellular localization of PLD2 and Grb2 was analyzed by indirect immunofluorescence. Cells were serum-starved overnight and then incubated with or without 100 ng/ml EGF for 5 minutes, fixed and analyzed for PLD2 and/or Grb2 using immunolabeled primary antibodies, followed by FITC or TRITC as secondary antibodies, respectively. **(A)** Endogenous expression and localization of PLD2 (upper panel) or Grb2 (lower panel) before (left) and after (right) EGF treatment. **(B)** Endogenous colocalization of PLD2 and Grb2. Uper panel, PLD2 expression; central panel, Grb2 expression and lower panel, merged images.