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PKC α Binds G3BP2 and Regulates Stress Granule Formation Following Cellular Stress

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Abstract

Protein kinase C (PKC) isoforms regulate a number of processes crucial for the fate of a cell. In this study we identify previously unrecognized interaction partners of PKC α and a novel role for PKC α in the regulation of stress granule formation during cellular stress. Three RNA-binding proteins, cytoplasmic poly(A)⁺ binding protein (PABPC1), IGF-II mRNA binding protein 3 (IGF2BP3), and RasGAP binding protein 2 (G3BP2) all co-precipitate with PKC α . RNase treatment abolished the association with IGF2BP3 and PABPC1 whereas the PKC α -G3BP2 interaction was largely resistant to this. Furthermore, interactions between recombinant PKC α and G3BP2 indicated that the interaction is direct and PKC α can phosphorylate G3BP2 *in vitro*. The binding is mediated via the regulatory domain of PKC α and the C-terminal RNA-binding domain of G3BP2. Both proteins relocate to and co-localize in stress granules, but not to P-bodies, when cells are subjected to stress. Heat shock-induced stress granule assembly and phosphorylation of eIF2 α are suppressed following downregulation of PKC α by siRNA. In conclusion this study identifies novel interaction partners of PKC α and a novel role for PKC α in regulation of stress granules.

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Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that play important roles in several processes that control cell fate such as apoptosis, proliferation, and differentiation. The PKC isoforms are grouped in classical (PKC α , β I, β II, and γ), novel (PKC δ , ϵ , η , and θ) and atypical (PKC ζ , and ι) PKCs [1]. Each isoform can be specifically regulated and has unique functions in a given cell. This is conceivably in part attributed to differential sensitivities to activating factors that are increased upon stimulation of cell surface receptors. For example, only classical isoforms are activated by Ca²⁺ and only classical and novel isoforms are sensitive to diacylglycerol. In addition to these differences in activator sensitivity, a wide range of studies indicate that isoform-specific interactions with other proteins largely determine the function of a PKC isoform [2].

In order to further understand PKC isoform function we have screened for interaction partners by immunoprecipitating a neuritogenic PKC ϵ structure and identified co-precipitated proteins by mass spectrometry analysis. One of these was the intermediate filament protein peripherin [3]. In addition, several proteins containing RNA recognition motifs (RRMs) were identified. These include the cytoplasmic poly(A)⁺ binding protein (PABPC1), the IGF-II mRNA binding protein 3 (IGF2BP3), and the RasGAP binding protein 2 (G3BP2).

PABPC1 binds to the poly(A) tail of mRNAs and its primary role is conceivably in regulating translation initiation and mRNA stability [4]. IGF2BP3 was first detected overexpressed in pancreatic cancer and initially called KOC (after *KH* domain

containing protein overexpressed in cancer) but later identified as an IGF-II mRNA-binding protein and re-named [5,6]. It is one of three members of the IGF-II mRNA-binding protein (IMP) family which are important for transporting their mRNA targets to proper cellular localization during development [7]. G3BP2 and the closely related protein G3BP1, which has been more studied, may have several roles in the cell. They have both been shown to interact with SH3 domains in GTPase-activating proteins [8,9] and with MYC mRNA [10] regulating its turnover. Furthermore, overexpression of G3BP1 leads to the assembly of stress granules which sequester and retain mRNA-species that are not supposed to be translated during the cellular stress response [11,12].

When analyzing endogenous proteins we could detect an interaction between PKC α and the three mRNA-binding proteins. The fact that all proteins contain RNA-binding domains, and that at least PABPC1 and G3BP are known to localize to stress granules, led us to raise the hypothesis that PKC may participate in stress granule formation. In this paper we demonstrate that PKC α both regulates the assembly of stress granules and is a component of them. The results therefore provide information regarding novel cellular roles of PKC α .

Results

PKC α interacts with G3BP2

In a screen for proteins that interact with an overexpressed PKC ϵ construct (PKC ϵ PSC1V3) [3], mass spectrometry analysis of some of the co-precipitated proteins resulted in the identification of the RNA-binding proteins IGF2BP3, PABPC1, and

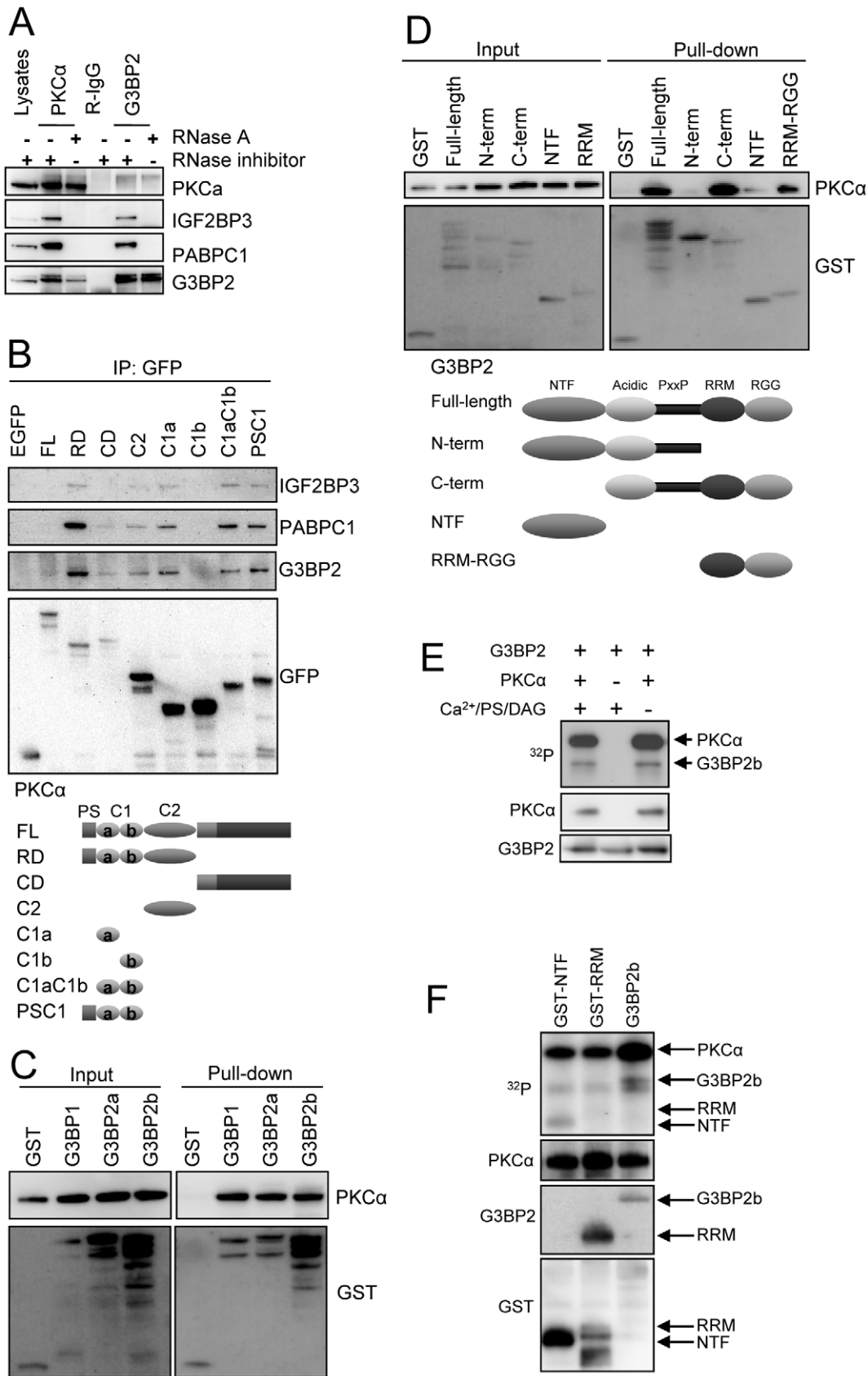


Figure 1. Interaction of PKC α with G3BP2, IGF2BP3 and PABPC1. (A) PKC α and G3BP2 were immunoprecipitated from SK-N-BE(2)C cell lysates that had been treated with RNase or a RNase inhibitor. Immunoprecipitates were thereafter subjected to Western blot with indicated antibodies. (B) SK-N-BE(2)C cells were transfected with vectors encoding EGFP fusions of full-length PKC α (FL) or the regulatory (RD), catalytic (CD), C2, C1a, C1b,

tandem C1a and C1b (C1ab), or PSC1 (PSC1) domains. The constructs are illustrated below the blot. Cell lysates were immunoprecipitated using anti-GFP-conjugated magnetic beads. Lysates and precipitates were thereafter analyzed with Western blot using IGF2BP3, PABPC1, G3BP2 or GFP antibodies. PKC α was incubated with GST-tagged G3BP1, G3BP2a and G3BP2b (C) or different G3BP2b constructs (D). Thereafter GST was pulled down with GSH-coupled sepharose. Incubation mixture and pull-downs were analyzed with Western blot. Schematic illustration of the G3BP2 constructs is shown below the blot. (E) G3BP2b (with GST cleaved off) was incubated with PKC α , [γ - 32 P]ATP and PKC activators (Ca $^{2+}$ /PS/DAG - Ca $^{2+}$, phosphatidylserine and diacylglycerol) as indicated. The reactions were separated on SDS-PAGE, blotted and [32 P] was visualized with autoradiography and proteins with immunoblotting. (F) G3BP2b (with GST cleaved off) and GST-tagged G3BP2 domains were incubated with PKC α , [γ - 32 P]ATP and PKC activators. The reactions were analyzed as in (E). doi:10.1371/journal.pone.0035820.g001

G3BP2. Although the interactions could be confirmed for overexpressed PKC α constructs we could not detect endogenous interactions under normal growth conditions. However, we found that the proteins interacted with PKC α (Fig. 1).

The fact that the identified PKC-interacting proteins also are RNA-binding proteins raised the possibility that the interaction is dependent on intact RNA. We therefore analysed the interaction in cell lysates that had been treated with either RNase inhibitor or RNase prior to immunoprecipitation (Fig. 1A). The interaction of PKC α with IGF2BP3 and PABPC1 was abolished by RNase treatment whereas G3BP2 and PKC α could still be co-precipitated with each other. G3BP2 itself interacted with IGF2BP3 and PABPC1 in an RNA-dependent manner, which could suggest that the proteins are in a common complex.

To analyze the structures in PKC α that mediate the interactions, EGFP-fusions of PKC α domains were expressed in SK-N-BE(2)C neuroblastoma cells and immunoprecipitated using the EGFP tag (Fig. 1B). G3BP2, PABPC1 and IGF2BP3 all co-precipitated with essentially the same PKC α constructs. The binding seems to be mediated primarily by the regulatory domain, where the C1a and C2 domains both have binding capability. On the other hand, the C1b domain did not interact with the proteins.

The data in Figure 1A indicate that there may be a direct binding between PKC α and G3BP2, whereas the association with IGF2BP3 and PABPC1 is indirect and dependent on RNA. Therefore we further analyzed the PKC α -G3BP2 interaction with a GST pull-down experiment with recombinant proteins (Fig. 1C). PKC α was pulled down together with GST-G3BP2 demonstrating a direct interaction between the proteins. Both G3BP2 variants (G3BP2a and G3BP2b which are generated by alternative splicing) bind PKC α . The binding is not specific for G3BP2 since the closely related protein G3BP1 also pulled down PKC α (Fig. 1C).

To examine which structures in G3BP2 that mediate the binding, GST-tagged domains of G3BP2 were included in the binding assay (Fig. 1D). The isolated RNA-binding domain pulled down PKC α whereas G3BP2 variants lacking this domain did not interact.

Taken together, the data suggest that the C1a and C2 domains in the regulatory domain of PKC α contain structures that can mediate the interaction with the C-terminal RNA binding domain of G3BP2.

We also analyzed if G3BP2 is a PKC substrate *in vitro* (Fig. 1E and 1F). G3BP2 was phosphorylated in the presence of PKC α , although not to the same extent as PKC α itself. The phosphorylation took place also in the absence of PKC activators (Fig. 1E). It is conceivable that the NTF, but not the RRM, domain contains PKC phosphorylation sites since GST-NTF but not GST-RRM was phosphorylated in the kinase assay (Fig. 1F).

A slower migrating PKC α variant is enriched in G3BP2 precipitates

As can be seen in Figure 1A the major PKC α species that is co-immunoprecipitated with G3BP2 does not migrate as fast as the major PKC α species in the PKC α immunoprecipitate. To certify

that this is PKC α and to investigate whether there is a difference in post-translational modifications of the PKC α variants, PKC α and G3BP2 immunoprecipitates were probed with different PKC α antibodies (Fig. 2). The upper band, enriched in G3BP2 precipitates, is clearly recognized by an antibody towards the N-terminal region of PKC α but more weakly by an antibody towards the C-terminal region. The antibody recognizing phosphorylated T638 identified both PKC α bands in a ratio similar as the general PKC α antibodies (Fig. 2A). However an antibody directed towards phosphorylated S657 primarily reacted with the upper band. Both antibodies towards phosphorylated PKC recognized the PKC α found in G3BP2 precipitates (Fig. 2B). Thus, the fact that several PKC α antibodies react with the slow-migrating species in the G3BP2 precipitate underscores that it is PKC α . G3BP2 primarily interacts with a PKC α variant that has both its C-terminal phosphorylation sites phosphorylated.

PKC α as well as G3BP2, IGF2BP3 and PABPC1 localizes to stress granules

PABPC1 and G3BP1 (closely related to G3BP2 and often referred to as G3BP) have previously been shown to be associated with the formation of stress granules during cellular stress [11,13]. We therefore investigated whether the three PKC-interacting proteins co-localize during stress. SK-N-BE(2)C cells were incubated at 44°C and PABPC1, G3BP2 and IGF2BP3 proteins were visualized by immunofluorescence (Fig. 3A). Under normal conditions PABPC1, G3BP2 and IGF2BP3 (not shown) are diffusely localized in the cytosol, whereas upon heat shock the proteins relocate to newly formed stress granules. Thus, the proteins co-localize in stress granules under these conditions.

To investigate whether PKC α accompanies the identified mRNA-binding proteins to stress granules SK-N-BE(2)C cells were subjected to heat shock and PKC α and PABPC1 were

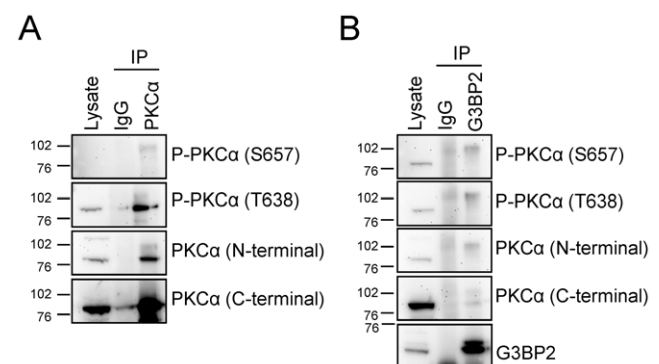


Figure 2. Analysis of the PKC α variant co-precipitated with G3BP2. Lysates from SK-N-BE(2)C neuroblastoma cells were incubated with either PKC α antibody (A) or G3BP2 antibody (B) and precipitated materials were separated by SDS-PAGE. Co-immunoprecipitated proteins were visualized with Western blot using antibodies indicated in the figure. P-PKC α , Phosphorylated PKC α . doi:10.1371/journal.pone.0035820.g002

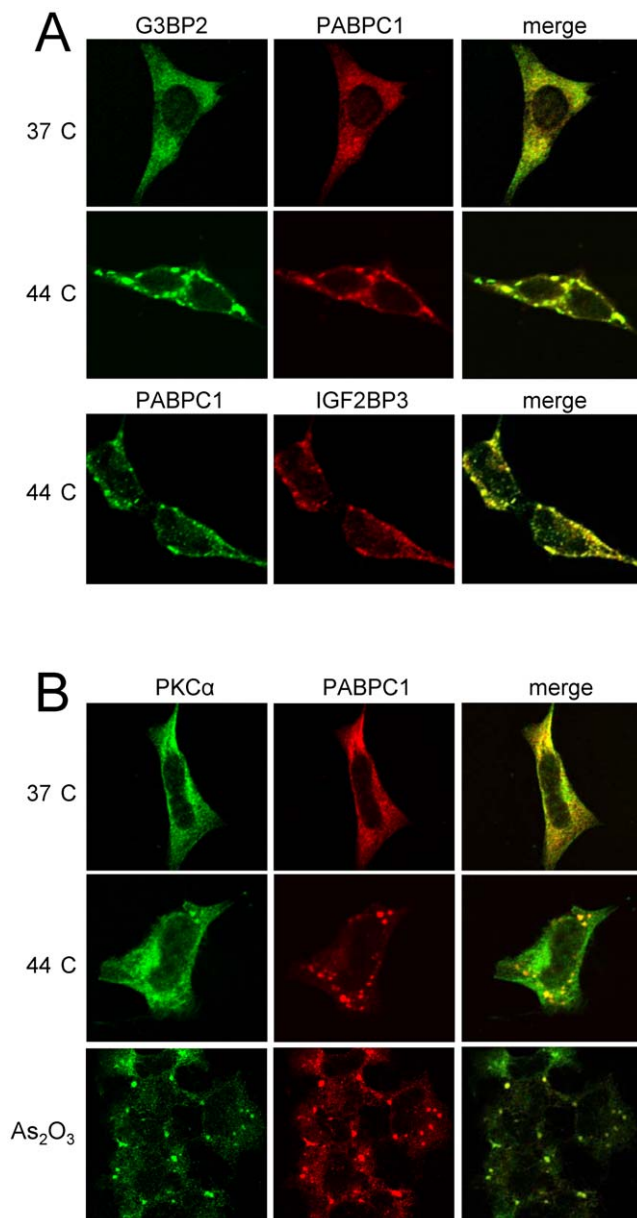


Figure 3. PKC α , G3BP2, PABPC1 and IGF2BP3 co-localize in stress granules. (A) SK-N-BE(2)C cells were either incubated at 37°C or heat shocked at 44°C for 1 h and endogenous G3BP2, IGF2BP3, and PABPC1 were visualized by immunofluorescence. (B) Cells subjected to heat shock or treated with 600 μ M of As₂O₃ for 1 h were analyzed with immunofluorescence towards PKC α . PABPC1 was used as stress granule marker. Cells were examined with confocal microscopy. doi:10.1371/journal.pone.0035820.g003

visualized by immunofluorescence (Fig. 3B). PKC α and PABPC1 both showed a diffuse cytosolic localization pattern in cells cultured at 37°C. After 1 h of heat shock PABPC1-containing stress granules were formed and PKC α was present in many of the PABPC1-containing granules (Fig. 3B). Accumulation of PKC α in PABPC1-containing stress granules was also observed after treatment with As₂O₃ (Fig. 3B) indicating that the PKC α relocation is not limited to the stress response induced by heat shock. A weak increase of PKC α reactivity in the nucleus could be seen after heat shock (Figs. 3B and 4A) but this was not the case following As₂O₃ treatment.

Processing-bodies (P-bodies) constitute another class of mRNA-rich granules that are functionally and spatially linked to stress granules [14,15]. To analyze whether PKC α also localizes to these structures, SK-N-BE(2)C cells were subjected to heat shock. PKC α and the P-body marker Dcp1a were thereafter visualized with immunofluorescence (Fig. 4A). PKC α could not be detected in Dcp1a-positive structures.

To simultaneously visualize P-bodies, stress granules and PKC α , the cells were transfected with a vector encoding EGFP-tagged PKC α and stained for G3BP2 and Dcp1a (Fig. 4B). Stress granules and P-bodies were in many cases localized immediately adjacent to each other with some overlapping pixels in the borders of the structures. However, PKC α displayed a clear co-localization with stress granules whereas isolated P-bodies were PKC α -negative.

The PKC α C1a domain associates with stress granule proteins after heat shock

Our data indicate that the C1a but not the C1b domain of PKC α contains structures that can mediate its interaction with G3BP2 (Fig. 1B). To investigate if it also can mediate the association with stress granule components, the PKC α C1a and PKC α C1b domains were expressed in SK-N-BE(2)C cells that were subsequently subjected to heat shock. To enrich for stress granule components we immunoprecipitated the stress granule component TIAR (Fig. 5). As expected, G3BP2 was co-precipitated with TIAR following heat shock, indicating that stress granule components are enriched in the precipitate. The experiment revealed that the PKC α C1a domain, but not the PKC α C1b domain, co-precipitates with TIAR upon heat shock, indicating that the C1a domain can mediate interaction with stress granule components.

Downregulation of PKC α delays stress granule formation

Since PKC α interacts with stress granule components and also localizes to these structures we postulated that PKC α may be involved in the regulation of stress granule formation. To test this hypothesis we aimed at downregulating PKC α with siRNA and study the stress granule induction. Due to difficulties in obtaining substantial knockdown of PKC α in SK-N-BE(2)C cells, we used the breast carcinoma MDA-MB-231 cell line for these experiments. PKC α localizes to stress granules upon heat shock also in MDA-MB-231 cells (data not shown). MDA-MB-231 cells were transfected with three different PKC α siRNA oligonucleotides and were thereafter subjected to a 44°C heat shock (Fig. 6A and 6B). Western blot demonstrated decreased PKC α levels following transfections with PKC α siRNA. Silencing of PKC α led to a decrease of the amount of cells with stress granules under heat shock (from 86% \pm 7% for control to 48% \pm 13 for siPKC α (I)-, 41% \pm 16% for siPKC α (II)-, and 49% \pm 14% for siPKC α (III)-transfected cells; Fig. 6A and 6B). PKC α downregulation did not delay the disassembly of the stress granules following a reversal of the temperature to 37°C.

The effects of PKC α downregulation on stress granules formation was compared with knock-down of PKC ϵ (Fig. 6C). Suppression of PKC ϵ levels did not influence the formation of stress granules as PKC α did. PKC α downregulation only suppressed stress granule formation during the initial phase suggesting that absence of PKC α does not abolish stress granules but rather delays their assembly. The As₂O₃-induced stress granule assembly was not significantly reduced in PKC α -downregulated cells, suggesting that the importance of PKC α depends on the stress inducer (Fig. 6D).

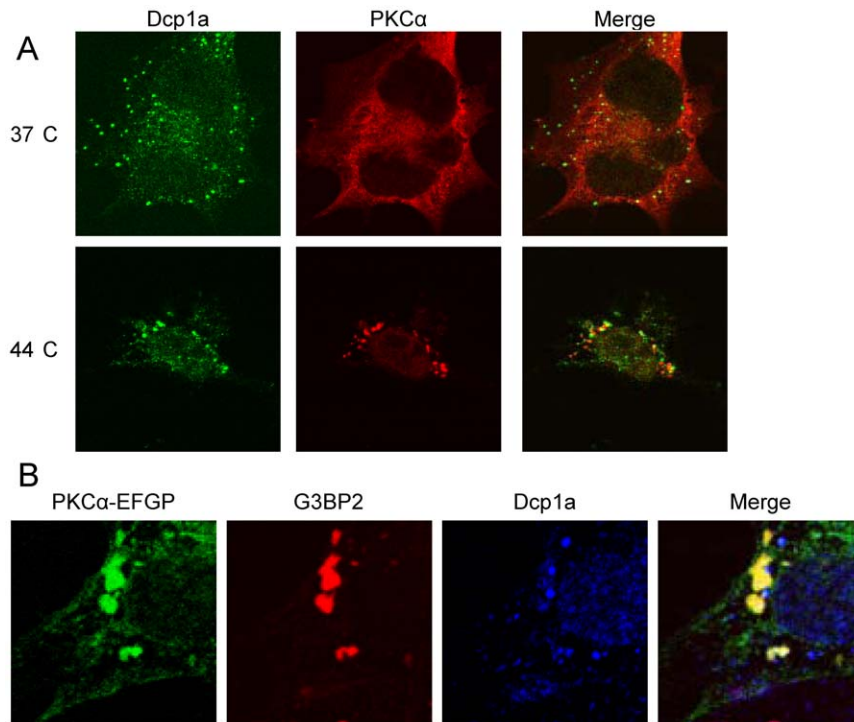


Figure 4. PKC α does not localize to P-bodies. Non-treated SK-N-BE(2)C cells (A) or SK-N-BE(2)C cells transfected with a vector encoding PKC α -EGFP (B) were placed at 44°C for 1 h. PKC α and the P-body marker Dcp1a (A) or Dcp1a and G3BP2 (B) were thereafter visualized by immunofluorescence. Cells were analyzed by confocal microscopy and in (B) PKC α was detected by the EGFP fluorescence. doi:10.1371/journal.pone.0035820.g004

To analyze whether PKC activity affects stress granule formation we treated cells with the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and/or the inhibitor GF109203X concomitantly with heat shock (Fig. 6E). Neither agent induced stress granules by themselves. However, the heat shock-induced stress granule formation was potentiated by the PKC inhibitor.

Downregulation of PKC α delays heat shock-induced phosphorylation of eIF2 α

Translational arrest by phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) is one of the major triggers that induce stress granule formation. We therefore analyzed if downregulation of PKC α also leads to suppression of the heat shock-induced eIF2 α phosphorylation (Fig. 7A and 7B). As for stress granule formation, there was a suppression of the initial phosphorylation of eIF2 α whereas after prolonged stress no effect of PKC α downregulation could be discerned. As₂O₃ exposure also leads to increased phosphorylation of eIF2 α . However, contrary to heat shock, we could not detect a suppression of the phosphorylation in PKC α -depleted cells (Fig. 7C and 7D).

To investigate whether downregulation of a kinase upstream of eIF2 α could explain the effect of PKC α depletion on heat shock-induced eIF2 α phosphorylation we analyzed the levels of protein kinase R (PKR) and heme-regulated inhibitor kinase (HRI), two kinases that may mediate eIF2 α phosphorylation during heat shock (Fig. 7E). PKR levels were, if anything, increased in PKC α -depleted cells. However, HRI levels were lower in PKC α -depleted cell in all four experiments but the p-value was not below 0.05. As a comparison we included lysates from PKC ϵ -depleted cells. The levels of HRI and PKR were not influenced as much in these cells.

To obtain further insights into PKC ϵ we analyzed whether PKC ϵ co-precipitates with IGF2BP3, PABPC1 and G3BP2 (Fig. 7F). We could not detect PKC ϵ in either precipitate under normal growth conditions. However, following heat shock, PKC ϵ could be discerned in IGF2BP3- and G3BP2 precipitates.

Discussion

Here we report for the first time that PKC α is a component of stress granules and that it associates with RNA-binding proteins G3BP2, IGF2BP3 and PABPC1. These findings provide novel information regarding PKC-mediated regulation of the cellular response to stress.

Since the identified PKC α interaction partners all are RNA-binding proteins it suggests a role for PKC α in RNA regulation. Indeed the interaction with IGF2BP3 and PABPC1 was dependent on intact RNA indicating the central role of RNA for the association. On the other hand, the interaction with G3BP2 was largely resistant to RNase treatment and could also be obtained with isolated proteins *in vitro*, indicating that it is a direct binding.

It has long been recognized that PKC can influence protein synthesis by acting at the RNA level. Several studies have demonstrated that PKC activation leads to increased stability of mRNA species [16–21]. The mechanisms by which PKC achieves this are still largely unknown but the mRNA-binding Hu proteins are one group of potential mediators. PKC regulates the Hu proteins both by increasing their expression levels [22] with subsequent stabilization of target mRNAs and by phosphorylation which influences its shuttling in and out of the nucleus [23,24]. A role for PKC in mRNA regulation is also supported by the identification of PKC β II in messenger ribonucleoprotein com-

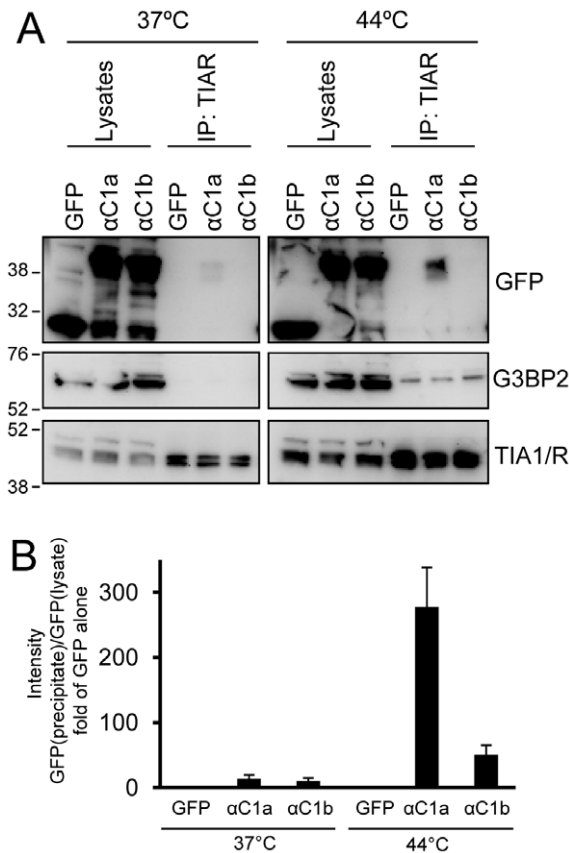


Figure 5. The PKC α C1a but not C1b domain is recruited to TIAR-containing complexes upon heat shock. SK-N-BE(2)C neuroblastoma cells were transfected with vectors encoding EGFP alone, or EGFP fused to the PKC α C1a or C1b domain and incubated at 37°C or 44°C for 1 h before harvesting. Cell lysates were sonicated and immunoprecipitated using anti-TIAR. Lysates and precipitates were analyzed with Western blot using antibodies indicated in the figure (A). The intensities of the EGFP fusions were quantified and the ratio of the intensity in precipitate to the intensity in the lysates is shown in (B). Data are mean \pm SEM, n=3. doi:10.1371/journal.pone.0035820.g005

plexes [25]. Upon activation, PKC β II binds RACK1 in the complex and RACK1 was shown to bind both PABPC1 and G3BP2.

The interaction with RACK1 is also important for PKC to modulate ribosomal subunit joining [26]. Our results show that PKC α interacts with the mRNA binding proteins in cells under basal conditions, and in the case of G3BP2 the interaction is direct, further highlighting that PKC α may have a role in post-transcriptional regulation in general.

Our data particularly support a role for PKC α in the regulation of mRNA that takes place during stress. When cells are exposed to stress, translation is shifted towards synthesis of proteins of importance for the cellular stress response. The translation of other mRNAs is temporarily silenced and they accumulate in stress granules, which contain the small ribosomal subunits, translation initiation factors, and a vast array of RNA-binding proteins [12,27]. The granules are dynamic and as soon as the cell is no longer exposed to stress stimuli they dissolve and protein translation is resumed [13]. Stress granules are formed when stress-sensitive serine/threonine kinases recognize and phosphorylate eIF2 α , an important component of the translation initiation complex [28]. Alternatively formation of stress granules can be

triggered when translation initiation is blocked at other steps such as inhibition of eIF4 or eIF4G activities or 80S ribosome assembly [29–31]. Stress granules can also be induced by overexpression of stress granule components, such as G3BP1 [11], T-cell intracellular antigen-1/T-cell intracellular antigen-related proteins (TIA-1/TIAR) [32], survival of motor neurons protein (SMN) [33], cytoplasmic polyadenylation-binding protein (CPEB) [34] and fragile X mental retardation protein (FMRP/FXR1) [35] in the absence of stress.

It is also becoming increasingly clear that stress granule assembly and disassembly are under the control of a diverse set of proteins that are not directly RNA-binding. Some act on modulating the post-translational modification of stress granule-related proteins. For example, stress granules are positive for ubiquitin [36] which apparently is of crucial importance since proteasome inhibition leads to stress granule formation [37] and the downregulation of the ubiquitin-binding protein HDAC6 suppresses their formation [36]. A functional screen revealed *O*-linked *N*-acetylglucosamine-modified proteins were enriched in stress granules and important for stress granule formation [38]. Phosphorylation of stress granule components also regulates the granules. Focal adhesion kinase (FAK)-mediated phosphorylation of Grb7 leads to its release from stress granules and is accompanied by stress granule disassembly [39] and phosphorylation of G3BP1 suppresses stress granule assembly by inhibiting its oligomerization [11]. Furthermore, the formation is microtubule-dependent [40] and potentiated following knockdown of apoptosis-inducing factors [41].

Our results, that PKC α relocates to these granules during stress and that knockdown of PKC α in MDA-MB-231 cells affects granule assembly after heat shock add PKC α to the list of stress granule regulators. PKC α depletion primarily led to a delay in the assembly which is analogous to the effect caused by depletion of importin α 1 or by interference with microtubules [42]. PKC α did not localize to P-bodies demonstrating that it is not associated with all mRNA containing granules but is more specifically involved in stress granule dynamics. The fact that simultaneous incubation with a PKC inhibitor did not suppress stress granule formation indicates that PKC α kinase activity is not directly involved in the pathway leading to stress granules. PKC isoforms have in other system been shown to exert effects independently of its kinase activity [43,44] and this may be another process regulated by PKC in a similar manner. Another alternative explanation could be that lower PKC α amounts during a longer time period may alter levels or functions of components important for stress granule formation. It is conceivable that the effects of PKC α depletion at least partially can be explained by alterations upstream of eIF2 α phosphorylation since this event was also delayed in PKC α -downregulated cells. It is possible that lower levels of the upstream eIF2 α kinase HRI is responsible for the suppressed heat shock-induced phosphorylation of eIF2 α in PKC α -depleted cells.

It is likely that the PKC α C1a domain is one mediator of the interaction since this domain, as opposed to the structurally similar C1b domain, was associated with the RNA-binding proteins and was enriched in TIAR precipitates after heat shock. This supports an interrelation between the PKC α interaction with G3BP2 and its localization to and role in stress granule formation. The C1 domains were originally identified as the binding sites for phorbol esters [45] but a number of studies have emerged showing they can mediate protein interactions that are both PKC isoform-specific [46,47] as well as common for several isoforms or C1 domain-containing proteins [48–50].

We could also see that G3BP2 preferably associates with a PKC α variant with a slower migration pattern, suggesting that,

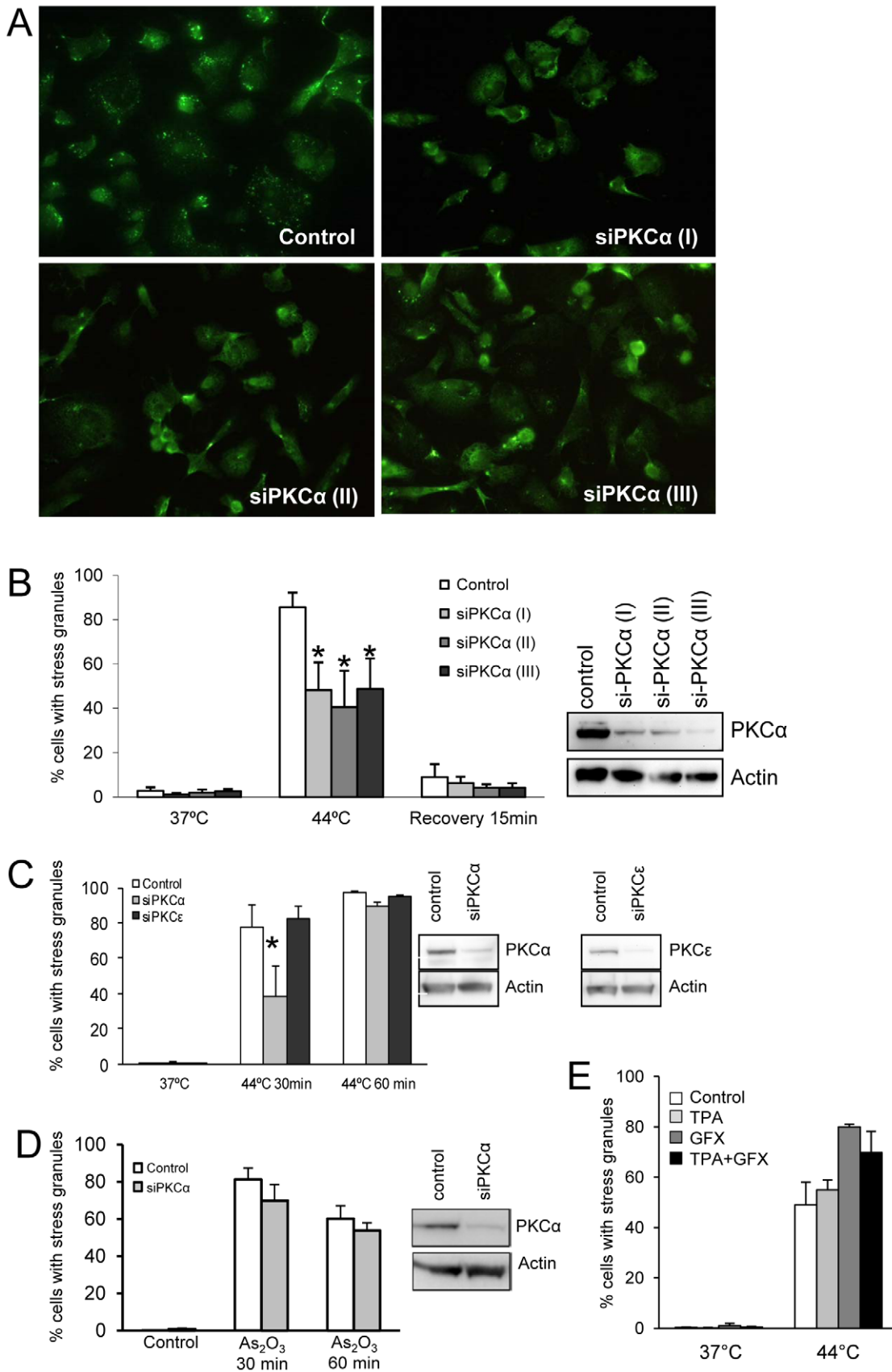


Figure 6. Downregulation of PKC α suppresses stress granule assembly. MDA-MB-231 cells were transfected with three different siRNA oligonucleotides against PKC α , and were thereafter subjected to heat shock at 44°C for 1 h. Stress granules were visualized with immunofluorescence

using a G3BP2 antibody (A). Western blot of cell lysates demonstrating PKC α downregulation and quantification of the percentage of cells containing stress granules (B) (mean \pm SEM, n=3). (C) MDA-MB-231 cells were treated with siRNAs targeting PKC α or PKC ϵ , followed by heat shock for indicated time periods. Cell lysates were analyzed with Western blot demonstrating downregulation of respective isoforms. Stress granules were visualized by G3BP2 immunofluorescence and the percentage of cells with stress granules was quantified (mean \pm SEM, n=3). (D) MDA-MB-231 cells with downregulated PKC α were treated with 300 μ M As₂O₃ for 30 or 60 minutes. Cell lysates were analyzed with Western blot and the percentage of cells with stress granules, identified by PABPC1 immunofluorescence was quantified (mean \pm SEM, n=3). (E) MDA-MB-231 cells were treated with 16 nM TPA and/or 2 μ M GF109203X (GFX) during heat shock. Stress granule-positive cells were thereafter quantified. * denotes statistically significant (p<0.05) difference compared to control using ANOVA followed by Duncan's multiple range test. doi:10.1371/journal.pone.0035820.g006

post-translational modification of PKC α is of importance for its association with G3BP2. Our analyses indicate differences in the phosphorylation pattern between the major PKC α variant and the one that is enriched in G3BP2 precipitates. One putative explanation to this difference is that a special conformation of PKC α is favorable for the interaction with G3BP2.

In conclusion the data demonstrate novel PKC α interaction partners which open up for mechanistic explanations of PKC α effects on RNA metabolism and stress granule-mediated regulation of the cellular response to stress.

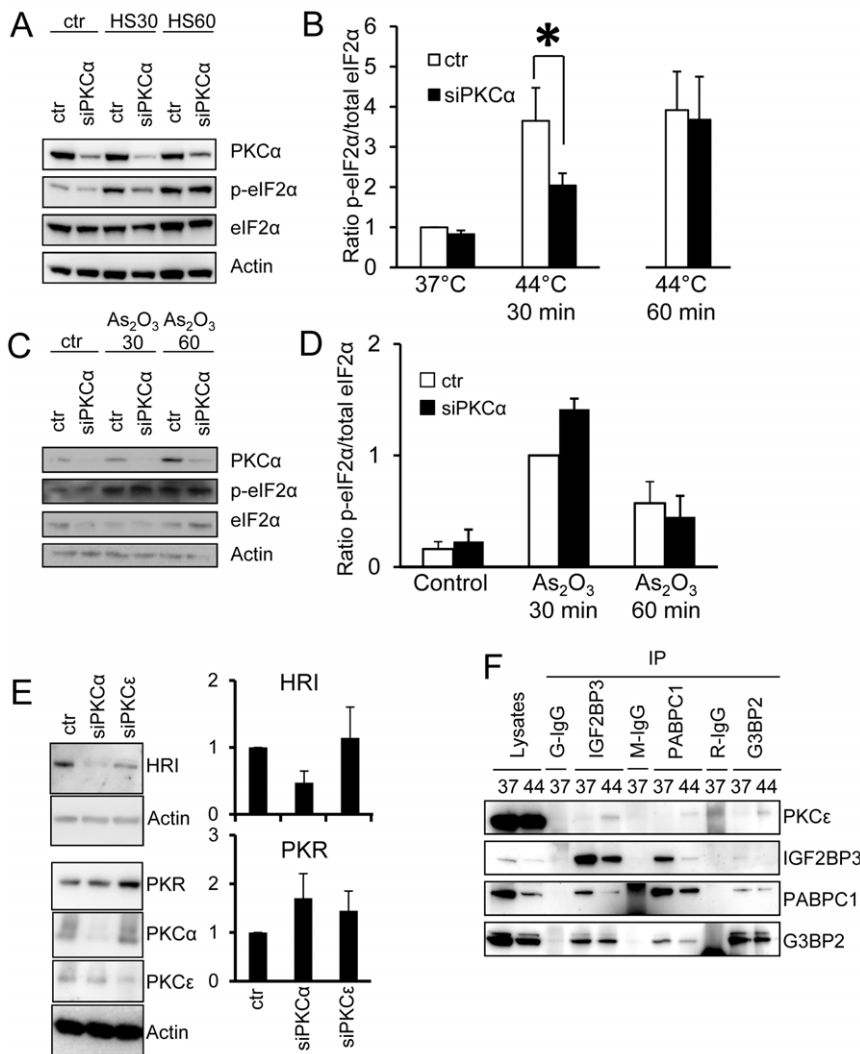


Figure 7. Heat shock-induced phosphorylation of eIF2 α is delayed in cells with downregulated PKC α . PKC α was downregulated in MDA-MB-231 cells by siRNA prior to subjection to heat shock (A and B) or As₂O₃ treatment (C and D) for indicated time periods. Lysates were analyzed for phosphorylated eIF2 α , total eIF2 α , PKC α and actin by Western blot (A and C). The levels were quantified and related to total eIF2 α and normalized to values obtained in control cells treated with a control siRNA (B and D) (mean \pm SEM, n=3). (E) PKC α and PKC ϵ were downregulated in MDA-MB-231 cells and the expression levels of HRI and PKR were analyzed with Western blot. The graphs show quantification of HRI and PKR levels divided by actin levels and normalized to control. Data are mean \pm SEM, n=4. (F) Lysates from cells that had or had not been subjected to heat shock were immunoprecipitated with antibodies towards IGF2BP3, PABPC1 and G3BP2 or matching isotype controls. The precipitates were thereafter analyzed for the presence of PKC ϵ . * denotes statistically significant (p<0.05) difference compared to control using ANOVA followed by Duncan's multiple range test. doi:10.1371/journal.pone.0035820.g007

Materials and Methods

Plasmids, antibodies and siRNA oligonucleotides

Expression vectors encoding full-length or isolated domains of human PKC α fused to enhanced green fluorescent protein (EGFP) have been described previously [43,51,52]. G3BP2b, G3BP2a and G3BP1 vectors were constructed by PCR of full-length templates (originally obtained from RZPD Deutsches Ressourcezentrum für Genomforschung GmbH for G3BP2b and cDNA from MDA-MB-231 cells for G3BP2a and G3BP1) introducing restriction enzyme sites adapted for cloning in pET41b vector. Primers used are listed in Table 1. All constructs were sequenced.

Rabbit polyclonal antibodies towards G3BP2 were generated by Agrisera (Vännäs, Sweden). The C-terminal polypeptide (CRGTGQMEGRFTGQRR) was used as immunogen and the resulting serum was affinity purified. GST, IGF2BP3, PABPC1, TIA1/TIAR (H-120), TIAR(C-18), HRI (S-16), and PKC α (antigen in C-terminus) antibodies were obtained from Santa Cruz, PKC α rabbit monoclonal antibody (antigen in N-terminus) from Epitomics, TIA1 and PKR from Abcam, phospho-PKC α / β II (pThr638/641), phospho-PKC (pan) (pSer660 - PKC β II), and eIF2 α antibody from Cell Signaling, phospho-eIF2 α (pSer52) antibody from Stressgen and Cell Signaling, GFP antibody from Zymed, GST antibody from Oncogene, and Dcp1a antibody from ABNOVA. Secondary horseradish peroxidase-conjugated antibodies were obtained from Amersham Biosciences and antibodies conjugated to Alexa dyes from Molecular Probes. Control IgG was from Santa Cruz (goat IgG), Jackson ImmunoResearch (rabbit IgG) and ImmunoKontakt (mouse IgG1).

Sequences of StealthTM siRNA oligonucleotides (Invitrogen) are listed in Table 1.

Cell culture, transfections, and stress treatments

SK-N-BE(2)C cells were grown in minimum essential medium (Sigma), whereas MDA-MB-231 cells were grown in RPMI 1640 medium (Sigma). All media were supplemented with 10% fetal bovine serum (EuroClone), 100 IU/ml penicillin (Gibco), and 100 μ g/ μ l streptomycin (Gibco). MDA-MB-231 medium was additionally supplemented with 1% sodium pyruvate (Gibco). Cells were kept at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Cells were seeded at densities of approximately 1.5–2 \times 10⁶ cells/100-mm, 3 \times 10⁵ cells/60-mm, and 1.5–2 \times 10⁵ cells/35-mm dishes. For siRNA transfections the cell density was halved. Transfections were initiated 24 hours after seeding and were performed with 2 μ g plasmid DNA and 2 μ l Lipofectamine 2000 (Invitrogen) per ml Optimem I medium (Gibco) according to the supplier's protocol. For siRNA transfections cells were incubated for 48 or 72 h with 40 nM oligonucleotides and 2 μ l/ml medium of Lipofectamine 2000 in Optimem. For induction of stress, cells were incubated at 44°C or treated with As₂O₃ (Sigma Aldrich) in a humidified atmosphere containing 5% CO₂ and 95% air. When indicated cells were incubated with 16 nM TPA and 2 μ M GF109203X (both from Sigma Aldrich).

Immunoprecipitation

Cells were treated as indicated in the protocol supplied with the μ MACS Epitope-Tagged Protein Isolation Kit (Miltenyi Biotec). Briefly, cells were washed twice in ice cold PBS and lysed for 30 minutes on ice in either a lysis buffer supplied with the kit (150 mM NaCl, 1% triton X-100, 50 mM Tris HCl, pH 8.0) (Figs. 1A, 2B, 5, and 7F) or a polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, 1 mM DTT, 1% IgePAL CA-630, pH 7.0) (Fig. 1B) for co-precipitation analyses, or in RIPA

Table 1. Sequence of oligonucleotides.

siRNA oligonucleotide	sequence
control 44% GC	GACAGUUGAACGUCGAUUUGCAUUG
control 48% GC	UUACGGAUCGACUUAAGCCGUUGCA
PKC α I	CCGAGUGAAACUCACGGACUCAAU
PKC α II	CCAUCGGAUUGUUCUUUCAUAA
PKC α III	UCCAAACGGCUUCAGAUCCUUAU
Primers for cloning	Sequence 5' to 3'
G3BP1	
Forward	GCGAGATCTAATGGTGATGGAGAAGCCTAG
Reverse	CGGCTCGACTCACTGCCGTGGCGAAGCCC
G3BP2 full length	
Forward	GCGAGATCTAATGGTTATGGAGAAGCCCAGT
Reverse	GCGGTCGACTCAGCGACGCTGTCTGTGAAG
G3BP2 N-terminus	
Reverse	CGGCTCGACTCAACTATCTGGATAGCGAATTAT
G3BP2 NTF2	
Reverse	CGGCTCGACTCAATCACCAAACTTCATCTTC
G3BP2 RRM-RGG	
Forward	GCGAGATCTAATTCGCTATCCAGATAGTCA
G3BP2 C-terminus	
Forward	GCGAGATCTAGAAGATGAAGTGTGGTGTGAT

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lysis buffer (10 mM Tris-HCl pH 7.2, 160 mM NaCl, 1% Triton X-100, 1% Na-deoxy-cholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA) (Figs. 2A, 6, 7A, 7C and 7E) for other experiments. The buffers were supplemented with CompleteTM protease inhibitor cocktail without EDTA (Roche). Lysates were cleared by centrifugation at 14,000 \times g for 10 min at 4 °C, and incubated either with anti-GFP-conjugated microbeads for 30 minutes or with 1 μ g of antibodies for 1 h to overnight prior to addition of protein G-coupled microbeads and an additional incubation for 30 minutes on rotation at 4 °C. The immune complexes were recovered by applying the lysates on μ Columns placed in the magnetic field of a μ MACS Separator. Following washes the complexes were eluted with sample buffer.

Western blot

Proteins were electrophoretically separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes were pre-incubated with 5% dried milk in PBS followed by incubation with primary antibodies. Membranes were washed, incubated with horseradish peroxidase-labelled secondary antibody, and immunoreactivity was detected with the SuperSignal system (Biological Industries), enhanced chemiluminescence detection system (GE Healthcare) or SuperSignal (Pierce) as substrate. The chemiluminescence was captured with a charge-coupled device camera (Fujifilm) and intensities were quantified with ImageJ.

GST pull-down

GST/His fusions of G3BP2 variants and G3BP1 were expressed in *Escherichia coli* BL-21(DE3) (Stratagene). Following induction for 4 hours with 1 mM IPTG, bacteria were lysed in buffer (50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 10 mM imidazol, 4% CompleteTM protease inhibitor cocktail without EDTA) and kept at -80°C overnight. Lysates were sonicated after addition of 1 mM DTT and 1 mg/ml lysozyme. Cleared lysates were mixed with Ni-NTA agarose (Quiagen) for one hour and applied on an EconoPac[®] Disposable Chromatography Columns (BIO-RAD). The agarose was washed twice with washing buffer (20 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazol, 1 mM DTT). His-tagged proteins were eluted in four fractions with 1.5 ml elution buffer each (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazol, 1 mM DTT).

GST pull-down assay was performed incubating 80 ng PKC α isozyme (Sigma) with 4 μ g of GST-G3BP recombinant proteins in 100 μ l binding buffer (20 mM Tris, pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT) with agitation for 1 hour at 4°C. Thereafter 40 μ l μ MACSTM anti-GST MicroBeads (Miltenyi Biotec) was added and following 1 hour incubation at 4°C, protein separation was performed on a μ Column in a magnetic field of μ MACS Separator (Miltenyi Biotec) according to manufacturers

protocol. GST pull-downs were analyzed with SDS-PAGE and Western blotting.

In vitro kinase assay

Since the GST/His fusion of G3BP2b has the same size as PKC α the GST/His tag was proteolytically removed with thrombin to enable identification on autoradiography. G3BP2b or GST/His fusion of G3BP2 domains (1 μ g) was incubated in with 400 ng PKC α (Sigma-Aldrich), 100 μ M (2 Ci/mmol) [γ -³²P]ATP (Perkin Elmer), 20 mM HEPES (pH 7.4) and 10 mM MgCl₂. Reactions were either supplemented with 0.5 mM EGTA (absence of activators) or with 0.3% Triton X-100, 100 μ g/ml phosphatidylserine (Sigma Aldrich), 0.1 mM CaCl₂ and 20 μ g/ml 1,2-diacylglycerol (Avanti). The total volume was 50 μ l. Reactions were incubated at 30°C for 20 min and terminated by addition of sample buffer. Samples were separated by SDS-PAGE and subjected to autoradiography and Western blot.

Immunofluorescence and confocal microscopy

Cells were washed in PBS, fixed with 4% paraformaldehyde in PBS for 4 minutes, washed twice in PBS and thereafter permeabilized and blocked with 5% goat serum or 5% bovine serum albumin and 0.3% Triton X-100 in PBS for 30 minutes. Cells were incubated with primary antibodies for 1 h. Following washes in PBS, cells were incubated with secondary Alexa Fluor 488-, 546-, and/or 633-conjugated antibodies in PBS for 1 h followed by extensive washes in PBS and mounting on object slides using 20 μ l PVA-DABCO (9.6% polyvinyl alcohol, 24% glycerol, and 2.5% 1,4-diazabicyclo[2.2.2]octane in 67 mM Tris-HCl, pH 8.0).

For confocal microscopy a Bio-Rad Radiance 2000 confocal system fitted on a Nikon microscope with a 60x/NA 1.40 oil lens or a Zeiss LSM710 was used. Excitation wavelengths were 488 nm (EGFP and Alexa Fluor 488), 543 nm (Alexa Fluor 546), and 637 nm (Alexa Fluor 633) and the emission filters used were HQ515/30 (EGFP and Alexa Fluor 488) and 600LP (Alexa Fluor 546). In triple stainings a HQ600/50 bandpass filter was used for Alexa Fluor 546 detection and a 660LP filter for Alexa Fluor 633. For quantification of stress granules 200 cells were scored for the presence of stress granules, identified either by PABPC1 or G3BP2 antibodies.

Author Contributions

Conceived and designed the experiments: TK SW LS CL. Performed the experiments: TK SW LS UH. Analyzed the data: TK SW LS CL. Wrote the paper: TK SW LS CL.

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