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PKC β II Modulates Translation Independently from mTOR and through RACK1

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SYNOPSIS

RACK1 is an abundant scaffolding protein, which binds active PKC β II increasing its activity *in vitro*. RACK1 has also been described as a component of the small ribosomal subunit, in proximity of the mRNA exit channel. We tested the hypothesis that PKC β II plays a specific role in translational control and verified whether it may associate with the ribosomal machinery. Here we find that specific inhibition of PKC β I/II reduces translation as well as global PKC inhibition, but without affecting phosphorylation of mTOR targets. These data suggest that PKC β II acts as a specific PKC isoform affecting translation in an mTOR-independent fashion, possibly close to the ribosomal machinery. By far-Western, we found that PKC β II binds ribosomes *in vitro*. Coimmunoprecipitation studies indicate that a small but reproducible pool of PKC β II is associated with membranes containing ribosomes, suggesting that also *in vivo* PKC β II may physically interact with the ribosomal machinery. Polysomal profiles show that stimulation of PKC results in increased polysomes/80S ratio, associated with a shift of PKC β II to the heavier part of the gradient. A RACK1-derived peptide that inhibits the binding of active PKC β II to RACK1 reduces polysomes/80S ratio and methionine incorporation, suggesting that binding of PKC β II to RACK1 is important for PKC-mediated translational control. Finally, downregulation of RACK1 by siRNA impairs the PKC-mediated increase of translation. Taken together these data show that PKC β II can act as a specific PKC isoform regulating translation, in an mTOR independent fashion, possibly close to the ribosomal machinery.

Heading title: mTOR independent translational control

Keywords: PKC; RACK1; initiation; phorbol esters; hispidin; eIF6

Abbreviations used: PKC, Protein Kinase C

INTRODUCTION

Translation requires at least 11 eIFs (eukaryotic Initiation Factors) controlling rate and accuracy [1]. The global initiation rate is controlled by signaling on the eIF4F complex, regulating cap-dependent translation, and on the eIF2 complex, bringing the ternary complex ($\text{tRNA}_i^{\text{Met}}$, eIF2 and GTP) on the 40S subunit [2]. The most studied stimulatory pathway on translation is through mTOR (mammalian Target Of Rapamycin) kinase that acts on the formation of the cap complex, by the phosphorylation of the negative regulator 4E-BP1 (eIF4E-Binding Protein 1) [3,4]. The mTOR pathway is activated by growth factors, and optimal nutrient conditions. However, in most cells mTOR inhibition by rapamycin blocks only 15% of the translation rate [5], suggesting that other pathways parallel to mTOR may be important. For instance, stimulation of translation by adhesion to extracellular matrix is totally independent from mTOR activation [6], and translational recovery after hypertonic stress requires Erk1/2 signaling [7]. Thus, dissecting mTOR independent pathways is of major importance, in order to fully grasp the complexity of translational regulation.

The protein kinase C superfamily includes different isoforms that are involved in most cellular processes, including cell cycle control, differentiation, and apoptosis. In mammals, eleven PKC isoforms exist, divided into three subgroups on the basis of their responsiveness to general PKC activators [8]. Conventional PKCs ($\text{PKC}\alpha$, $\text{PKC}\beta$ /II, $\text{PKC}\gamma$) require both diacylglycerol (DAG) and Ca^{2+} for activation. They can also be activated by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), in a Ca^{2+} -dependent manner. Novel PKCs ($\text{PKC}\delta$, $\text{PKC}\epsilon$, $\text{PKC}\eta$, $\text{PKC}\theta$) are DAG/PMA sensitive but do not respond to Ca^{2+} . Finally, atypical PKCs ($\text{PKC}\zeta$ and $\text{PKC}\iota/\lambda$) are unresponsive to DAG and Ca^{2+} and are activated by interaction with other signaling proteins.

PKCs have long been thought to be involved in translational control, on the basis of the observation that, in general, PKC stimulation by pharmacological agents leads to increased translation [9]. In principle, PKC-mediated control of translation can either be due to activation of pathways upstream of initiation factors, namely through the PI3K/Akt/mTOR and MAPK/S6K pathways [10], or to the direct phosphorylation of initiation factors and ribosome-associated proteins. As an example of indirect activation of the translational machinery, $\text{PKC}\eta$ acts both on the activation of the PI3K, mTOR cascade [11] and the S6K phosphorylation [12], therefore triggering eIF4F complex formation. Increased levels of eIF4F then stimulate cap-dependent translation. The direct phosphorylation of components of the translational apparatus by PKC is less clear-cut. Several reports have identified among initiation factors and ribosome-associated factors, potential substrates of PKC, *in vitro*, but their unequivocal demonstration, *in vivo*, is lacking. A case is the one of eIF4E that is efficiently phosphorylated by PKC, *in vitro*, [13] and is found phosphorylated upon PKC activation, *in vivo* [14]. However, knockout studies showed that Mnk1 and Mnk2 (MAP kinase interacting kinases 1 and 2) are the responsible kinases, *in vivo* [15]. Similarly, *in vitro* phosphorylation by PKC has been reported for the elongation factor eEF1 [16], and recently for other proteins associated with the translational machinery such as eIF6 [17]. This said, the issue of direct substrates of PKC on the ribosomal machinery, *in vivo*, is complicated by the existence of multiple PKC isoforms differentially expressed and by the fact that it is unclear whether PKCs and ribosome-associated substrates can be in close proximity. In this context, the requirement of membrane-derived DAG for PKC activation brings a positional constraint for the phosphorylation of ribosome-associated factors, and suggests that PKCs may act directly on the ribosomal machinery exclusively in the presence of adaptors or intermediate molecules, or in proximity of membranes. It is therefore important to verify whether specific PKC isoforms can copurify with ribosomes or membrane-ribosome complexes. One putative adaptor between PKC and ribosomes is RACK1.

RACK1 (Receptor for Activated C Kinase) is a conserved protein homologous to the β -subunit of heterotrimeric G proteins. Its sequence contains seven internal Trp-Asp 40 (WD40) domains, devoted to protein-protein interaction. WD40 domains are predicted to fold into a β -propeller

structure, each domain forming a blade of the propeller [18,19]. RACK1 has been identified as a receptor for PKC, after its activation by DAG and Ca^{2+} [20]. Subsequent work has shown that the preferred PKC isoform(s) binding RACK1 are PKC β s [21]. The PKC β locus generates two isoforms by alternative splicing, PKC β I and PKC β II. Both bind RACK1, although PKC β II at higher affinity. Intriguingly, PKC β II is highly expressed in hypertrophic heart [22,23], where translational rates are pathologically increased. RACK1 is very abundant and can function as a general adaptor molecule, binding other membrane-associated proteins such as Src kinase, integrin β 1, the NMDA receptor [24].

Work in yeast and mammalian cells have demonstrated that RACK1 (Asc1p in yeast) binds the small ribosomal subunit in the cytosol [25] and mRNPs [26]. A similar situation has been observed in fission yeast, where Cpc2/RACK1 copurifies with the small subunit [27]. RACK1 also binds eukaryotic initiation factor 6 (eIF6), and *in vitro*, in the presence of PKC, can mediate the release of eIF6 from the ribosome [17]. These data suggested that RACK1 might function as a PKC adaptor on the ribosomal subunit. More recently, a cryo-EM map of the eukaryotic ribosome has shown that RACK1 is located in proximity of the mRNA exit channel, in close contact with the binding surface of eIF3 complex, and close to mRNA binding proteins [28]. Thus, RACK1 may provide a platform for allowing PKC translocation to ribosomes, and a subsequent direct action of PKC on the translational machinery.

We tested the hypothesis that RACK1 acts as a PKC β kinase platform on ribosomes by a) defining the effect of the pharmacological inhibition of PKC β II on translation, b) characterizing the RACK1/PKC β II interaction on the ribosomal apparatus, c) studying the effect of inhibition of RACK1/PKC β II binding, d) studying the effect of exogenous PKC β II overexpression. We report that PKC β II modulates global translational rate and provide evidence supporting a model in which PKC β II/RACK1 interaction has a role in PKC-based stimulation, independent from mTOR.

MATERIALS AND METHODS

Reproducibility

All immunoprecipitations, metabolic assays and polysomal profiles were performed at least three times, with similar outcomes. For immunoprecipitations and polysomal profiles, representative experiments are shown.

Cell lines and treatments

HeLa and HEK293 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), glutamine and antibiotics. Transfections were carried out with either calcium phosphate (HEK) or Gene Porter Transfection Reagent (HeLa) according to manufacturer instruction (GTS). All stimulations were performed after 3-6 hours growth factor starving. Cells were either stimulated with 50 nM PMA, 100 nM insulin for 30 min, or pre-treated with 50 μ M LY294002, 100 nM BMI, 5 μ M hispidin, 50 nM rapamycin 15 min before stimulation.

Drugs and reagents

The following antibodies were used: rabbit polyclonal anti-phospho-Akt (Ser 473), anti-phospho-S6 (Ser 235/236) and anti-rpS6 (Cell Signaling); rabbit polyclonal isoform-specific anti-PKC β II (C-18) (Santa Cruz); mouse monoclonals anti-PKC ϵ , anti-RACK1, anti-PKC β (BD Transduction Laboratories), anti-KDEL (Stressgen), anti- β actin (Sigma). Rabbit polyclonal anti-4E-BP1 was from N. Sonenberg, McGill University, Montreal, Canada [29]. Rabbit polyclonal anti-human β 1 integrin was from I. De Curtis, San Raffaele Scientific Institute, Milano, Italy [30]. Rabbit polyclonal anti-rpS14 was from T-A. Sato, Columbia University, New York, USA [31]. Plasmids: PKC β II from A. Newton [32]. PMA (phorbol 12-myristate 13-acetate), insulin (human, recombinant), and cycloheximide were from Sigma. The PKC inhibitor BMI (Bisindolyl Maleimide I), PKC β inhibitor hispidin, mTOR inhibitor rapamycin and BSA (bovine serum albumin) were from Calbiochem, PI3K inhibitor LY294002 was from Alexis Pharmaceuticals. RNasin[®] was from Promega.

In vitro binding assay of PKC β II

The *in vitro* binding of PKC β II to ribosomes was performed as follows. Five pmol purified ribosomal subunit 40S and 60S [33] and 10 ng recombinant PKC β II and PKC ϵ (CalBiochem) were applied on nitrocellulose membrane (Biorad). The membrane was blocked for 1 h with 5% (w/v) defatted milk in phosphate-buffered saline containing 0.05% (v/v) Tween 20. Membranes were probed with 100 μ l of lysate from HEK293 cells, which had been diluted 1:1 with lysis buffer containing 5% (w/v) defatted milk. This step was performed 1 hr RT. Further development of the blots was done exactly as for standard Western blots.

The overlay assay of PKC β II with RACK1 was performed as previously described [20] with some modifications. Recombinant RACK1 (125 ng) was blotted onto nitrocellulose membrane (Schleicher & Schuell) using a slot blot apparatus (Schleicher & Schuell). Unbound material was removed, and the membrane was incubated in overlay block (0.2 M NaCl, 50 mM Tris-HCl pH 7.5, 3% bovine serum albumin, and 0.1% polyethylene glycol) for 1 h at room temperature. In a separate tube, PKC β II (1 μ g of purified recombinant SF9-expressed protein) was incubated in the presence of 50 μ g/ml phosphatidylserine, 0.8 μ g/ml DAG, and 1mM calcium in overlay buffer (50 mM Tris-HCl pH 7.4, 0.1% bovine serum albumin, 5 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor 0.1% polyethylene glycol, 0.2 M NaCl, 0.1 mM CaCl₂, and 12 mM β -mercaptoethanol) and in the presence or absence of the peptide. The mixture was incubated for 15 min while being rotated at room temperature and then added to immobilized RACK1 for additional rotation of 15 min. Unbound material was removed, and the membrane was washed three times in overlay wash buffer (50 mM Tris-HCl pH 7.4, 0.1% polyethylene glycol, 0.2 M NaCl, 0.1 mM CaCl₂, and 12 mM β -mercaptoethanol). Binding of PKC β II was detected using anti-PKC β II polyclonal antibody followed by enhanced chemiluminescent reaction (Amersham Pharmacia Biotech) and processed using the STORM system (Molecular Dynamics).

Polysomal profile and fraction analysis

Polysomal profile was performed as described [17] with some modifications. Cells were pre-treated 10 minutes with 100 µg/ml cycloheximide, scraped in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 30 mM MgCl₂, 0.1% Igepal, 100 µg/ml cycloheximide, 40 U/ml RNasin[®], protease inhibitor cocktail (Sigma). Whole cell extracts were clarified at 4 °C 10 minutes 15,000 g. Ten OD₂₅₄ were layered on a 15-50% sucrose gradient in 50 mM Tris-acetate pH 7.5, 50 mM NH₄Cl, 12 mM MgCl₂, and 1 mM DTT and centrifuged at 4 °C in a SW41Ti Beckman rotor for 3.30 h at 39,000 rpm. Absorbance at 254 nm was recorded by BioLogic LP software (BioRad) and fractions collected. Fractions were precipitated with 10% trichloroacetic acid (TCA) according to standard protocol, separated on SDS-PAGE and analysed by Western blot. Densitometric analysis was performed by ImageQuant 5.0 software, Molecular Dynamics.

Permeabilization and metabolic assay

Subconfluent HEK293 cells grown in 35 mm diameter plates were washed with 1 ml ice-cold HBSS-H (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 1.03 mM MgSO₄, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5.6 mM glucose, 20 mM HEPES-NaOH pH 7.4) and then treated 1 min with 50 µg/ml saponin in 1 ml ice-cold ICM buffer without magnesium (see below) in the presence of 10 µM peptide. Cells were recovered for 5 min in 1 ml ice-cold ICM buffer (125 mM KCl, 19 mM NaCl, 10 mM HEPES-KOH pH 7.3, 0.33 mM CaCl₂, 1.4 mM MgCl₂) containing 10 µM peptide. Cells were incubated at 37 °C with serum-free medium for 2 hrs and with methionine-free medium for additional 2 hrs. Cells were pulsed with 28 µCi per plate of Promix ³⁵S-labelled methionine (Amersham) and stimulated with 100 nM insulin and 50 nM PMA for 30 min. Cells were lysed by scraping in 50 µl RIPA buffer without SDS (10 mM Tris-HCl pH 7.5, 1% Na-deoxycholate, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, proteases inhibitor cocktail). Ten µl extracts were TCA-precipitated on glass microfiber filters (Whatman) and counted. Obtained values were normalized on sample protein content, quantified by BiCinchonic Acid (BCA) protein assay (Pierce). Each sample was performed in triplicate and expressed as mean ± SEM. Statistical analysis was performed by ANOVA test.

Immunoprecipitation and Western Blot

Adherent subconfluent HeLa and HEK293 cells were lysed with 10 mM NaCl, 10 mM MgCl₂, 10 mM TrisHCl pH 7.3, 1% Triton X-100, 1% Na-deoxycholate, 1 mM DTT, 5 mM NaF, 2 mM Na₃VO₄, 40 U/ml RNasin[®], protease inhibitor cocktail. The whole cell extract was clarified at 4 °C at 15,000 g for 10 minutes. The amount of protein recovered was quantified by the BCA protein assay. For immunoprecipitation, 3 mg of total proteins were pre-cleared with protein A beads (Amersham), and then incubated for 2 h at 4 °C with anti-PKCβII/anti-PKCβ or with an irrelevant antibody. Protein A beads were added and incubated for 1 h at 4 °C. The beads were washed four times with lysis buffer and resuspended in sample buffer. Immunoprecipitates were resolved on SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with appropriate antibodies.

Spleen cells were derived from adult C57 mice as follows. Mice were sacrificed according to the procedures approved by the Institutional Animal Care and Use Committees (IACUC), and spleens were immediately dissected out. Finely minced spleens were forced through a 40 µm-sieved cell strainer in serum-free RPMI medium (Gibco). Cells were spun down, washed in serum-free RPMI, lysed and immunoprecipitation performed as described as above.

Phospho-protein detection was carried out in total lysates prepared in 20 mM HEPES pH 7.4, 100 mM NaCl, 1% Na-deoxycholate, 1% Triton X-100, 2 mM Na₃VO₄, 50 mM NaF, 10 mM β-glycerophosphate and protease inhibitor cocktail. The lysate was cleared by centrifugation. Protein content was quantified with BCA as above. Equal amount proteins were analyzed by Western blot.

Peptides design

R7 peptide (RRFVGHTKDV) was designed on the RACK1 sequence binding PKCβII. R9 peptide (GYTDNLIRVWQVT) was derived from the 7th WD40 repeat of RACK1. Peptides were synthesized as previously described [34]. Both peptides used in this study were over 90% pure.

Moreover, R7 peptide was made twice (the second one from Calso Laboratory, Lyngby, Denmark), and each batch gave the same biological activities.

RNA interference

The siRNAs targeted to human RACK1 was purchased from Ambion. The sequences are as follows: siRNA identification ID #135615 (siRNA1), 5'- CCAUCAAGCUAUGGAAUACTT-3' (sense) and 5'-GUAUUGCAUAGCUUGAUGGTT-3' (antisense); siRNA ID #135616 (siRNA2), 5'-GCUAUGGAAUACCCUGGGUTT-3' (sense) and 5'-ACCCAGGGUAUUGCAUAGCTT-3' (antisense); and siRNA ID #135617 (siRNA3), 5'-CCUUUACACGCUAGAUGGUTT-3' (sense) and 5'-ACCAUCUAGCGUGUAAAGGTG-3' (antisense). The siRNA negative control was purchased from Ambion (negative control siRNA #1). 1.5×10^5 cells were seeded per plate (35 mm diameter) the day before transfection, and then transfected with 50 nM oligonucleotide using 2 μ l Lipofectamine2000 Reagent (Invitrogen). The siRNA-lipofectamine mixture was prepared in Opti-MEM medium (Gibco) and added to cells. After 5 hours the cells were added with normal growing medium. Protein expression was assessed by Western blotting at 48 hours post-transfection and densitometric analysis was performed. Metabolic assay was carried out on siRNA transfected cells as described.

RESULTS

PKC β isoform can control translational rate in an mTOR-independent fashion

In HEK293 cells, PKC stimulation by PMA causes increase of protein synthesis and phosphorylation of 4E-BPs [9]. Indeed, polysomal profile analysis of PMA stimulated HEK293 cells, shows always augmented polysomal peaks and reduced 80S, indicating an increase at the initiation step of translation (Fig. 1A), accompanied by increased methionine incorporation (Fig. 1B). To dissect the underlying PKC pathway, we applied BMI, a broad spectrum PKC inhibitor [35] or hispidin, an isoform-specific PKC β inhibitor [36]. In conditions of stimulation, both PKC inhibitors cause decreased methionine incorporation (Fig. 1B). Interestingly, PKC β inhibition by hispidin represses methionine incorporation as efficiently as global PKC inhibition, suggesting its isoform-specific involvement. The inhibition of either PKC or of mTOR has no effect on non-stimulated cells indicating that the effect we see is specific for the conditions of stimulation (Supplementary Fig. 1).

Phorbol ester stimulation of all conventional PKCs causes activation of the ERK-S6K pathway and phosphorylation of 4E-BP1 [37]. Then we analyzed the effect of global PKC inhibition and specific PKC β inhibition on the phosphorylation status of 4E-BP1 and rpS6, which are downstream targets of mTOR and RSK/S6K [10], respectively. Broad PKC inhibitor BMI abolishes PMA-stimulated rpS6 and 4E-BP1 phosphorylation (Fig. 1C, lane 5), but not insulin-induced phosphorylation while PKC β inhibitor hispidin does not (Fig. 1C, lane 6). Thus PKC β isoform inhibition impairs translation without affecting the phosphorylation of mTOR targets. In agreement, the mTOR inhibitor rapamycin marginally represses PKC-stimulated translation by PMA, while it represses insulin-stimulated translation more effectively (Fig. 1D). Taken together these data indicate that PKC β affects translational efficiency, acting either downstream of the PI3K-Akt-mTOR axis or through a parallel pathway. Since PKC β binds RACK1, one possibility is that PKC β acts in proximity to ribosomes; thus, we studied the relationships between PKC β and ribosomes.

PKC β II associates with ribosomes

We first tested whether active PKC β II binds ribosomes directly. We spotted native 40S ribosomal subunits containing RACK1 or 60S subunits on filters, and performed binding with two PKC isoforms, β II and ϵ . PKC ϵ is a PMA sensitive, Ca²⁺ unresponsive PKC isoform that binds monomeric RACK1 [38]. We found that PKC β II specifically bound 40S subunits, but not 60S subunits, whereas PKC ϵ bound neither of them (Fig. 2A).

We measured whether PKC β II may transiently interact with ribosomes *in vivo*. By polysomal profiles, in unstimulated conditions, little or no PKC β II was found to co-sediment with the part of the gradient containing rpS6 (Fig. 2B, left panel). Upon PKC stimulation, we observed a reproducible shift of part of PKC β II towards fractions containing ribosomes (Fig. 2B, right panel, compare with RACK1 and rpS6 containing lanes). The increase of ribosome-associated PKC β II was two-fold (Fig. 2C), and specific for PKC β II since PKC ϵ never co-sedimented with ribosomes (Fig. 2B). Note that polysomal profiles were obtained in native conditions, without crosslinking, suggesting that the shift of PKC β II is comparable to the shift observed for some initiation factors [39]. This said, the absence of PKC β II from polysomes indicates that PKC β II is not retained during the elongation phase, whereas RACK1 is.

To further study the association of PKC β II with ribosomes, we immunoprecipitated endogenous PKC β II from unstimulated and PMA-stimulated cells, and tested for the presence of ribosomal proteins. PKC β II coimmunoprecipitates ribosomal protein rpS6 after stimulation (Fig. 2D). The amount of PKC β II-coprecipitated ribosomal proteins was low, but detergent-resistant. The association of ribosomes with PKC β II was also observed in mouse tissues, e.g. in the mouse spleen, an organ containing high levels of the kinase (Fig. 2E). The coprecipitation data show that *in vivo*, PKC β II has the potential to translocate to either ribosomes or ribosome-containing membranes.

Since ribosomes can normally associate to ER membranes, reportedly even in the absence of ongoing translation [40,41], we studied whether PKC β II activation resulted in translocation to membranes, including the endoplasmic reticulum. Indeed, PKC β II activation results in its translocation to endomembranes and E.R. (Suppl. Fig. 2 and [42]). Overall data in Fig. 1 and 2 lead to the hypothesis that PKC β II affects translation without acting on mTOR pathway, but preferentially interacting with the ribosomal machinery, either directly or through RACK1 or upon translocation to endomembranes.

RACK1-PKC β II complex modulates translation

An important issue is to define whether the binding of PKC β II to RACK1 is instrumental to PKC β II action on ribosomes and whether it may explain the localization of PKC β II on ribosomes. To investigate the significance of PKC β II and RACK1 association on translation, we used a RACK1-derived peptide (R7), which mimics the RACK1 binding surface to PKC β II and a control peptide (R9), which is designed on a RACK1 domain not involved in PKC β II binding. *In vitro* binding assay of PKC β II on immobilized RACK1 showed that R7 inhibits the association between active PKC β II and RACK1 in a concentration-dependent manner, while R9 is ineffective (Fig. 3, second and third couple bars).

Polysomal profiles of cells treated with R7 peptide, which blocks PKC β II binding to RACK1, showed a reduction in polysomal peaks and less ribosome-associated PKC β II, compared to R9 (Fig. 4A and B), without obviously affecting the localization of RACK1. We decided to approach the question whether the change in PKC β II distribution was associated with changes in methionine incorporation, too. Introduction of R7 blocking peptide in HEK293 cells caused a statistically significant reduction of PKC stimulated translation, whereas R9 did not (Fig. 4C; Fig. 6C). To further extend these data, we performed a series of experiments in which after treatment with R7/R9 peptides, polysomal profiles were run, mRNA was extracted from polysomal fractions and blotted for the presence of different mRNAs. We found that treatment with the R7 peptide reduced the polysomal association of several mRNAs (Suppl. Fig. 3), consistently with the effects on methionine incorporation.

Hispidin-based PKC β II inhibition caused a reduction in methionine incorporation without inhibition of mTOR activity. We tested whether R7-mediated impairment of translation caused an inhibition of mTOR activity. We found that R7 represses translation (Fig. 4C) without affecting rpS6 and 4E-BP1 phosphorylation (Fig. 4D, lane 5), exactly as hispidin (see Fig. 1B-C). If RACK1 is important in mediating PKC effects, reduction of RACK1 by siRNA may also affect PKC stimulated translation. We tested the hypothesis by RACK1 siRNA. A 70% reduction in RACK1 expression by siRNA (Fig. 4E) impaired responsiveness to PKC-mediated increase of translation (Fig. 4F).

Exogenously expressed PKC β II affects translation inhibited by RACK1-PKC β II disruption

Exogenous PKC β II recapitulated the results observed in HEK293 cells. PKC β II was overexpressed in HeLa cells, which express negligible endogenous PKC β II (Fig. 5A). The integrity and activation property of transfected PKC β II in overexpressing cells was tested by co-immunoprecipitation with RACK1 after activation (Fig. 5B), and by phosphorylation of a specific PKC substrate [43] (not shown). In HeLa overexpressing PKC β II, stimulation of PKCs resulted in increased cosedimentation of PKC β II with RACK1 containing fractions (Fig. 5C-D). We immunoprecipitated overexpressed PKC β II and blotted for the endogenous ribosomal protein of the 40S subunit rpS14 (Fig. 5E). A small, reproducible pool of rpS14 was associated with PKC β II, similarly to what observed for rpS6 in HEK293 cells. Exogenous overexpression of PKC β II led to increased methionine incorporation in newly translated proteins (Fig. 6A), while mock and PKC α (Fig. 6B) had no effect. Last, we measured translation in cells overexpressing PKC β II with administration of either R7 or R9 peptides. As we observed in HEK293, the stimulation of PKC resulted in increased

methionine incorporation in cells without peptides, or cells incubated with the R9 control peptide, but not in cells treated with R7 peptide (Fig. 6C).

DISCUSSION

One of the most studied pathway regulating translation is the mTOR dependent pathway, which acts mainly through 4E-BP1 phosphorylation. However, as rapamycin-mediated block of mTOR and translation in most cells blocks only 15% of translation, other pathways must exist. Here we have shown that PKC β II, at least in HEK293 and HeLa cells, affect translation through an mTOR independent pathway. Our data suggest that this pathway acts close to the ribosomal machinery through RACK1. The most consistent model explaining our data is that upon activation, PKC β II fulfills its membrane requirement by translocating to endomembranes, and then through its interaction with RACK1 can get in physical contact with the translational apparatus. Alternatively, coprecipitation of PKC β II with ribosomes is due to the fact that they may both reside on membranes (see below).

Structural and biochemical work has indicated that PKC β II binds RACK1 with nanomolar affinity, only after PKC activation [44]. The high affinity of binding, the high levels of RACK1 in cells, are consistent with the hypothesis that the RACK1-PKC complex can form in cells, in spite of the transient activation of PKC. The question is whether a RACK1/PKC complex can associate on the ribosome, *in vivo* and how stable it is. Several aspects must be taken in account: although *in vitro* PKC β II binds at high affinity RACK1 which in turns binds 40S, the detection of a stoichiometric interaction of PKC β II with ribosomes *in vivo* may be complicated by the kinetics of the interaction, possibly too rapid. Furthermore, PKC β II and ribosomes must be in equilibrium with endomembranes to allow PKC activation. Our model is that signaling to the translational machinery occurs at the interface between membranes and ribosomes, and RACK1 acts as a transient scaffold for PKC on the ribosome. In this context, we like to suggest that the presence of endomembranes may be a general feature of translational control: for instance the translational regulator mTOR-raptor complex, mTORC1, is detergent-sensitive. In addition, work from Nicchitta's lab [40,41] suggests that most ribosomes are membrane-associated even when translation is blocked. Another alternative is that a specific pool of RACK1, not ribosome-bound, interacts with the activated PKC β II, and then mediates a signal to the translational machinery. In this context the mixture RACK1/PKC is effective in mediating phosphorylation-dependent eIF6 release from 60S *in vitro* [17].

A question to be addressed is which ribosome-associated substrates of RACK1-PKC β II may account for PKC β II-induced translational activation. One substrate that is phosphorylated by RACK1-PKC complex is eIF6 [17]. However, the precise contribution of the activity of eIF6 in the control of translational rate, *in vivo*, is still unclear, due to its pleiotropic action on ribosome biogenesis [45,46], although eIF6 knockout mice have a translation deficit downstream of PMA stimulation (Gandin et al., in preparation). Furthermore, PKC activation results in a strong increase of translation in conditions of overexpression of eIF6 [17], and an increase in phosphorylated eIF6 [47]. As most other kinases, it is likely that RACK1-PKC β II complex may act on other substrates too, which remain to be characterized. Indeed, purified ribosomes show a sustained PKC-like activity (Grosso, unpublished results). Among the putative substrates, eIF4B has a PKC consensus sequence that is phosphorylated *in vivo*.

In conclusion, we provide evidence that a PKC isoform-specific pathway, alternative to the mTOR-4EBP1-S6K cascade, signals to the complexity of initiation factors and mRNA binding proteins acting on translational control.

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LEGENDS

Fig. 1. PKC β modulates translation without affecting the phosphorylation of mTOR targets. (A) Polysomal profiles of HEK293 cells, unstimulated (left panel) or stimulated with PMA (right panel). Initiation of translation (polysomes) increases upon PKC stimulation. (B) HEK293 cells were treated as indicated and methionine incorporation was counted. The experiment was carried out in triplicate. PMA-insulin mediated translation is inhibited both by the broad PKC inhibitor BMI and by PKC β specific inhibitor hispidin. (C) HEK293 cells were treated as described and analysed for anti-P-Akt (upstream factor in insulin cascade stimulating translation), anti-4E-BP1 and anti-P-rpS6 (markers for mTOR and S6K1 activation). Anti-rpS6 is used as a loading normalizer. LY294002 inhibits PI3K/mTOR-dependent translation. Note that hispidin impairs translation without affecting the phosphorylation pattern of mTOR targets. (D) HEK293 cells were treated as indicated and methionine incorporation was counted. Note that rapamycin inhibition does not impair PMA dependent translation.

Fig. 2. Endogenous PKC β II is associated to ribosomes. (A) Purified 40S and 60S ribosomal subunits were spotted on nitrocellulose membrane. Overlay assay of PKC β II over immobilized ribosomes shows preferential association with 40S subunit. Recombinant PKC β II and PKC ϵ (novel PKC isoform) were spotted as a control for isoform specificity of PKC antibodies. Note that increased staining of recombinant PKCs is due to their denaturation, whereas ribosomes and PKC on ribosomes is in native form (B) Fractions from polysomal profiles of HEK293 cells, unstimulated (left panel) or stimulated with PMA (right panel) were probed for PKC β II, PKC ϵ , RACK1 and rpS6. PMA treatment shifts PKC β II to the heavier part of the gradient. (C) Densitometric analysis of the percentage of PKC β II cosedimenting with ribosomes by pooling data from independent experiments. Data are expressed as the percentage of the total amount of PKC β II. rpS6-containing fractions define ribosome-bound PKC β II. (D) PKC β II was immunoprecipitated from unstimulated or stimulated HEK293. Input is 10% of IP. Immunocomplexes were probed for PKC β II and rpS6 as a ribosomal marker. (E) The same experiment as in (D) was carried out on mouse spleen. (D) and (E) show co-immunoprecipitation of ribosomes with PKC β II.

Fig. 3. R7 inhibits RACK1-PKC β II binding *in vitro*. Overlay assay of PKC β II on immobilized RACK1 was performed in presence or absence of R7 and R9 peptides as indicated. Phosphatidylserine (PS), diacylglycerol (DAG) and Ca²⁺ physiologically activate PKC β II. R7 prevents the binding of PKC β II to RACK1 in concentration dependent manner.

Fig. 4. Inhibition of RACK1-PKC β II binding affects translation. (A) HEK293 cells were permeabilized in presence of R7 or R9 peptide and then fully stimulated. The ratio between 40S+60S+80S monosomes (in white) and translating polysomes (in grey) is indicated as an inset. R7 peptide reduces polysomes vs. monosomes ratio. (B) HEK293 cells overexpressing PKC β II were permeabilized in presence of R7 or R9 peptide and then stimulated as in (A). Fractions from polysomal profile were immunoblotted as indicated. Note that R7 peptide prevents PKC β II loading on ribosomes. (C) Methionine incorporation in HEK293 cells. Data are from independent experiments carried out in triplicate. ANOVA test for statistic significance is indicated. (D) Same as in Figure 1C, using peptides R7 and R9, where indicated. R7 peptide impairs translation without affecting the phosphorylation pattern of mTOR targets. (E) Cells transfected with RACK1 siRNA or control. Densitometric analysis confirms reduction of RACK1 levels. PKC β II expression is not modified by RACK1 downregulation. (F) Cells transfected with RACK1 siRNA or control as in (E), were PMA-insulin stimulated or left untreated and metabolically labeled. Data from triplicate independent samples.

Fig. 5. Exogenous PKC β II associates with ribosomes. (A) Levels of endogenous and transfected PKC β II in HeLa cells. (B) RACK1 co-purifies with PMA-activated PKC β II. Input is 10% of IP. (C-D) Polysomal profiles from unstimulated (C) and PMA-stimulated (D) HeLa cells prepared as in Figure 1A. Fractions were probed for PKC β II and RACK1. The asterisk marks a PMA-dependent PKC β II degradation product. (E) PKC β II pull-downs were immunoprobed for PKC β II and for rpS14 as a ribosomal marker. Input is 10% of IP.

Fig. 6. Exogenous PKC β II affects translation through RACK1 binding. (A) Methionine incorporation in either mock-transfected or PKC β II-transfected HeLa cells. (B) As in (A) but with transfected PKC α . PKC β II transfection increases translational rate, whereas PKC α does not. (C) R7 reduces translation as compared to R9 (control peptide). Experiment carried out as in Fig. 3C. Grey bars: PMA-stimulation; white bars: basal. Data were obtained from triplicate independent samples.

Fig. 1

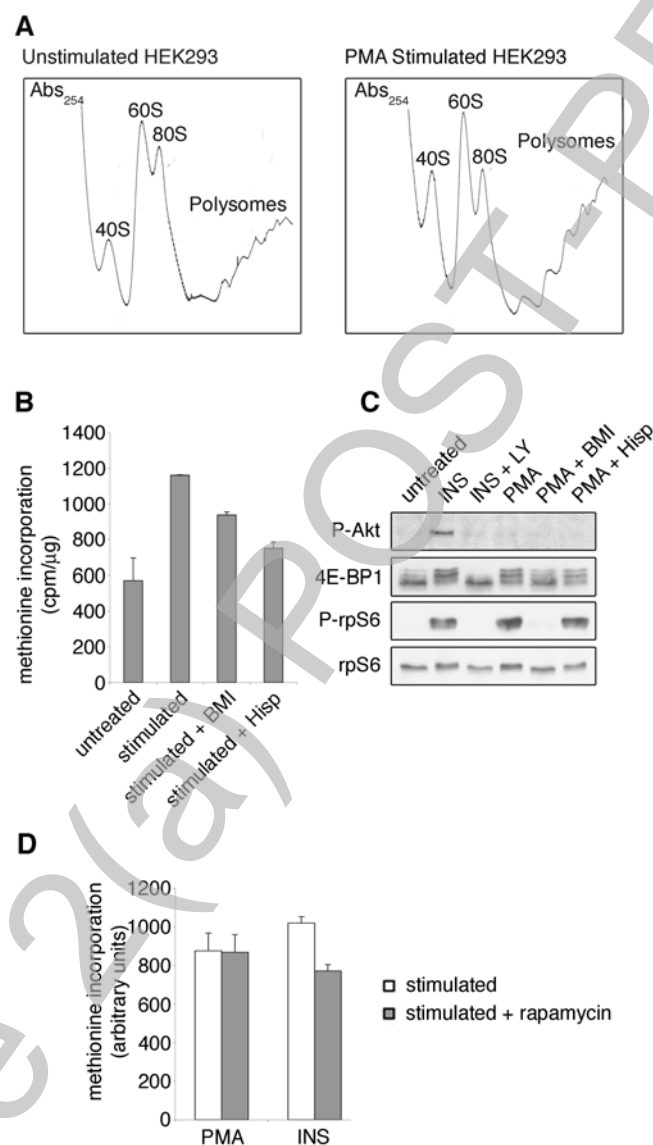
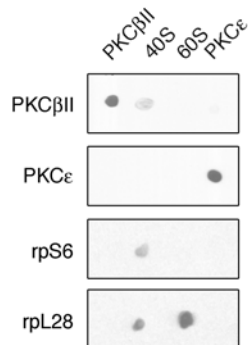
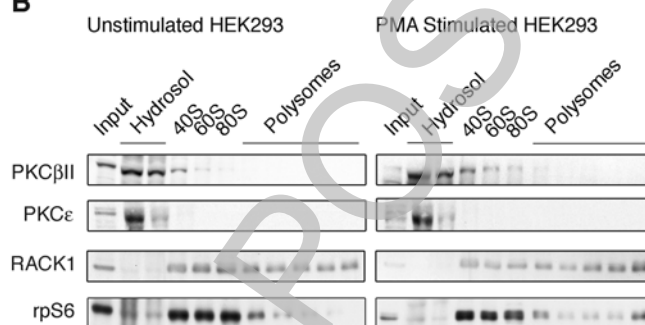


Fig. 2

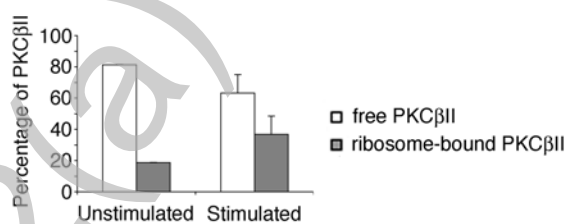
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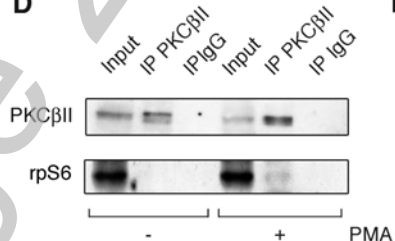
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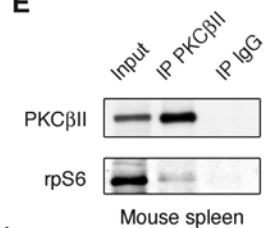


Fig. 3

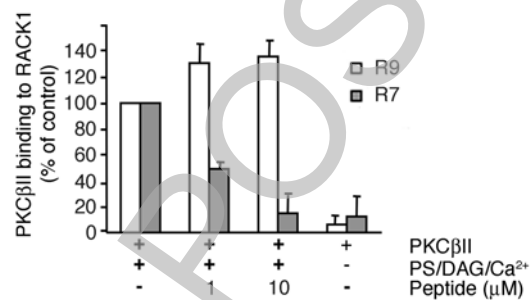


Fig. 4

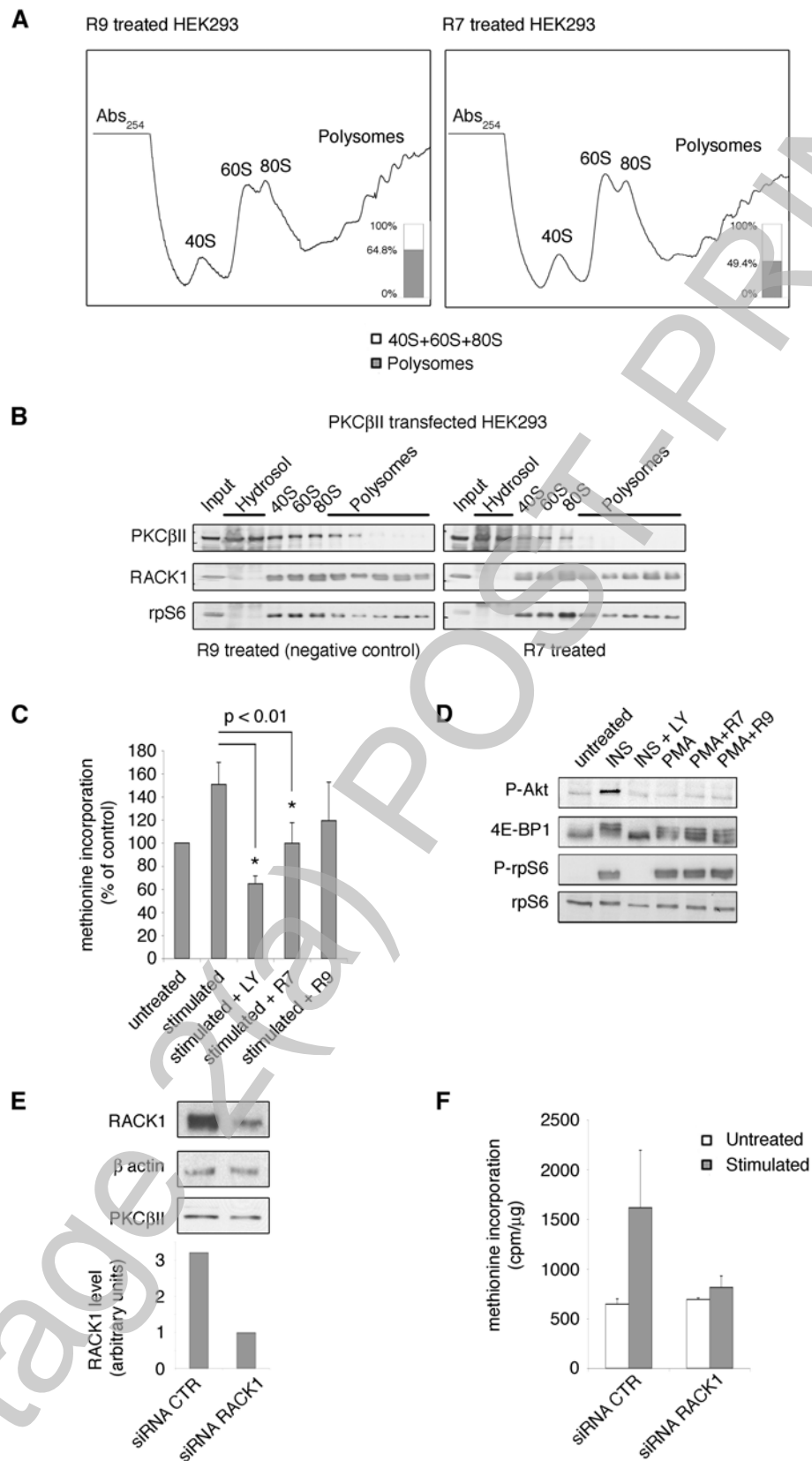


Fig. 5

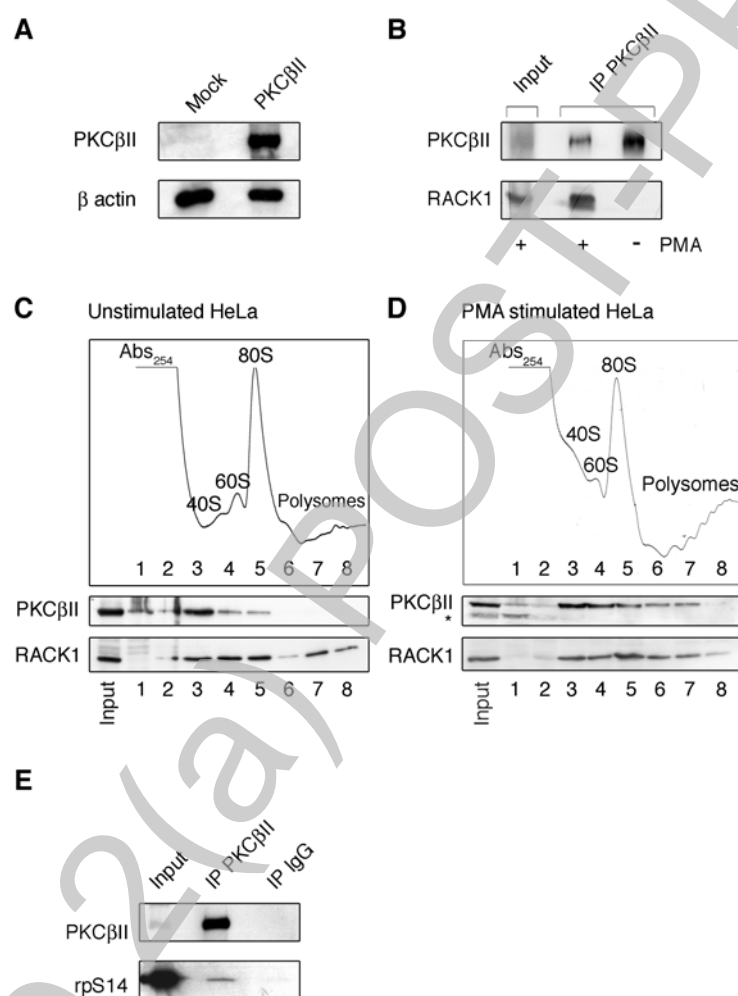


Fig. 6

