



## **Ras activation in response to phorbol ester proceeds independently of the EGFR via an unconventional nucleotide-exchange factor system in COS-7 cells**

Ignacio Rubio, Knut Rennert, Ute Wittig, Katrin Beer, Matthias Dürst, Stacey L. Stang, Jim Stone, Reinhard Wetzker

### **► To cite this version:**

Ignacio Rubio, Knut Rennert, Ute Wittig, Katrin Beer, Matthias Dürst, et al.. Ras activation in response to phorbol ester proceeds independently of the EGFR via an unconventional nucleotide-exchange factor system in COS-7 cells. *Biochemical Journal*, 2006, 398 (2), pp.243-256. <10.1042/BJ20060160>. <hal-00478523>

**HAL Id: hal-00478523**

**<https://hal.science/hal-00478523v1>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Ras activation in response to Phorbol ester proceeds independently of the EGFR via  
an unconventional nucleotide exchange factor system in COS-7 cells

**Ignacio Rubio<sup>1,4\*</sup>, Knut Rennert<sup>1,4</sup>, Ute Wittig<sup>1</sup>, Katrin Beer<sup>2</sup>, Matthias Dürst<sup>2</sup>, Stacey L. Stang<sup>3</sup>, Jim Stone<sup>3</sup> and Reinhard Wetzker<sup>1</sup>**

<sup>1</sup>Institute of Molecular Cell Biology, Medical Faculty, Friedrich-Schiller-University Jena, Drackendorfer Str.1, 07747 Jena, Germany; <sup>2</sup>Department of Obstetrics and Gynecology, Friedrich-Schiller-University Jena, Bachstrasse 18, 07743 Jena, Germany; <sup>3</sup>Department of Biochemistry, University of Alberta, Edmonton, AB, Canada.

Running title: Mechanism of Ras activation by Phorbol ester

<sup>4</sup>These authors contributed equally to this work

\* Address correspondence to this author:

Tel. + 49 (0)3641 9325670

Fax. + 49 (0)3641 9325652

E-mail: [b5igru@rz.uni-jena.de](mailto:b5igru@rz.uni-jena.de)

Abbreviations used: BIM, bisindolylmaleimide I; bp, base pairs; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR; EGF receptor; GAP, GTP hydrolase activating protein; GEF, guanine nucleotide exchange factor; GTPase, guanine nucleotide binding protein; HB-EGF; heparin binding EGF-like growth factor; HP, horseradish peroxidase; IAA, iodoacetic acid; LPA, lysophosphatidic acid; NAC, N-acetyl-cysteine; PE, Phorbol ester; PTP, protein tyrosine phosphatase; RBD, Ras binding domain of c-Raf1 kinase; ROS, Reactive Oxygen Species; TLC, thin layer chromatography; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

## Synopsis

Ras is a major mediator of Phorbol ester (PE) effects in mammalian cells. Various mechanisms for PE activation of Ras have been reported, including pathways that target GTP hydrolase activating proteins (GAPs) for inactivation and those that result in activation of guanine nucleotide exchange factors (GEFs) Sos or RasGRP. However, a biochemical link between PE and GAP inactivation is missing and GEF stimulation is hard to reconcile with the observation that dominant-negative S17NRas does not compromise Ras-dependent Erk activation by PE. We have addressed this controversy and carried out an in-depth biochemical study of PE-induced Ras activation in COS-7 cells. Using a cell-permeabilization approach to monitor nucleotide exchange on Ras, we demonstrate that PE-induced Ras-GTP accumulation results from GEF stimulation. Nucleotide exchange stimulation by PE is prevented by PKC inhibition but not by EGFR blockade, despite the fact that EGFR inhibition aborts basal and PE-induced Shc phosphorylation and Shc/GRB2 association. In fact, EGFR inhibition ablates basal nucleotide exchange on Ras in growth-arrested COS-7 cells. These data disclose the existence of two separate GEF systems that operate independently from each other to accomplish PE-dependent formation of Ras-GTP and to maintain resting Ras-GTP levels, respectively. We document that COS-7 cells do not express RasGRP and present evidence that the PE-responsive GEF system may involve PKC-dependent phosphorylation of Sos. More fundamentally, these observations shed new light on enigmatic issues such as the inefficacy of S17NRas in blocking PE action or the role of the EGFR in heterologous agonist activation of the Ras/Erk pathway.

Keywords: phorbol ester, Ras, nucleotide exchange, EGFR, Sos, RasGRP

## Introduction

Activation of K-, H- and N-Ras guanine nucleotide binding proteins (GTPases) (collectively referred to as Ras hereafter) results from an increase in the velocity ratio of two opposing reactions: the exchange of nucleotides on Ras resulting in GTP-loading as catalyzed by GEFs and the hydrolysis of Ras-bound GTP to GDP promoted by GAPs. Hence, investigation of the biochemical mechanisms by which extracellular agonists elevate Ras-GTP levels boils down to elucidating how they affect GEF and/or GAP activities.

The tumor promoter and diacylglycerol analogue Phorbol-12-myristate-13-acetate (TPA) activates Ras in a limited number of cell types, including cardiac myocytes, COS fibroblast-like cells, T24 bladder carcinoma cells and leukocytes. Ras activation in response to PE in all these cell types is sensitive to PKC inhibition [1-6] but beyond this consensus reported mechanisms differ substantially. In T-cells PE was originally proposed to down-regulate GAP activity via PKC, based on the finding that PE did not enhance the rate of nucleotide uptake by Ras in permeabilized cells [1]. PKC phosphorylates the p120GAP protein *in vitro* [7], providing a potential, albeit not yet proven biochemical link for this scenario. Similar conclusions have been drawn for COS and T24 bladder carcinoma cells based on the observation that PE activation of the Ras-effector Erk pathway was not affected by dominant-negative S17NRas [4,5,8,9]. Since S17NRas is believed to exert its blocking action by sequestering Ras-GEFs, these findings have been inferred to reinforce the notion that PE does not engage GEFs but rather down-regulates GAP activity [4]. In T24 cells, intracellular sphingosin-1-phosphate as produced by PKC-activated sphingosine kinase has been put forward as a link between PE and Ras activation [4]. However, how this pathway ultimately feeds into Ras and whether or not this process is mediated by GAP inhibition is not known.

Opposed to this view, several lines of evidence argue for an involvement of Ras-GEFs in PE signalling to Ras. Recent biochemical and genetic evidence has made a strong case for members of the RasGRP family of Ras-GEFs as mediators of PE-induced Ras activation in lymphocytes [6,10-12]. RasGRP GEFs interact physically with diacylglycerol or PE via their C1 domain, and hence represent direct target proteins for PE. Mature T-cells from RasGRP1<sup>-/-</sup> knockout mice show dramatically reduced formation of Ras-GTP in response to PE [11], strongly arguing for RasGRP1 as a major mediator of PE and diacylglycerol-elicited signalling to Ras. Analogous data from B-cells have shown that the B-cell receptor couples to the Ras/Erk pathway by means of the related GEF RasGRP3 [6,12]. Most recently, we have described the phosphorylation and concomitant activation of RasGRP1 and 3 by PKC, suggesting that PE feeds into RasGRP both through direct interaction

and indirectly via PKC [6,13]. The appeal of this scenario resides in that it provides a rationale for the well-established requirement for PKC in PE activation of Ras. However, RasGRP expression is largely limited to brain and lymphocytes [10,11,14], suggesting that this mechanism is unlikely to account for PE effects in other cell types.

Yet another line of evidence indicates that PE does access the Shc/Grb2/Sos pathway via PKC-mediated transactivation of the EGFR. PE induces tyrosine phosphorylation of the EGFR and Shc in a number of cell types [15-20]. In COS cells this pathway proceeds via metalloproteinase-mediated shedding of latent membrane-bound heparin binding EGF-like growth factor (HB-EGF) [15], which consequently transactivates the EGFR in an auto/paracrine fashion. Available data on the role of the EGFR in PE signalling to the Ras/Erk pathway are controversial. EGFR has been reported to mediate PE activation of Ras or Erk in some cell types [16,18,21], yet fails to do so in other systems or in the hands of other investigators [22-24]. Moreover, while GEF (Sos or RasGRP) engagement by PE is documented in a number of settings it is difficult to harmonize with the inefficacy of S17NRas in blocking PE activation of the Ras/Erk pathway, as observed in the majority (but not all [25,26]) of the cases where this has been investigated [4,5,8,9,27]. In summary, despite the documentation of a number of alternative mechanisms for PE-induced Ras activation, no singular pathway has been delineated as yet for a particular cell type.

In the present study we investigated PE activation of Ras in more depth by looking at the actual rate of nucleotide turnover on Ras in permeabilized cells. We observed that PE elevates guanine nucleotide turnover on Ras in COS epithelial cells, demonstrating that PE engages Ras-GEFs to activate Ras. We present evidence for the existence of two Ras-GEF systems that operate independently from each other: one responds to PE-activated PKC and mediates agonist-induced acceleration of nucleotide exchange, the other is fuelled by basal EGFR activity to maintain steady-state Ras-GTP levels in resting cells.

## **Experimental Procedures**

### Materials

Streptolysin O, the non toxic diphtheria-mutant CRM197, poly(Glu<sub>4</sub>Tyr), iodoacetic acid, N-Acetyl-L-Cysteine, bisindolylmaleimide I, GDP, GTP, heparin-agarose, hydrogen peroxide and DMSO were purchased from Sigma-Aldrich (Munich, Germany). AG1478 was from Merck Biosciences (Bad Soden, Germany). TPA and PD98059 were from Alexis Biochemicals (Lausen, Switzerland).

[ $\alpha$ -<sup>32</sup>P]GTP (3000Ci/mmol) was from NEN Life Science Products (Brussels, Belgium). Glutathione- and GammaBind-Sepharose were from Amersham-Biosciences (Freiburg, Germany).

### Antibodies

Rabbit polyclonal serum 176 was raised against an N-terminal peptide of RasGRP1 which is conserved in mouse and human [10]. The L247-9 RasGRP3 polyclonal serum was obtained from rabbits immunized with a fragment of human RasGRP3 encompassing residues 544 to 689 [6]. These sera recognize both human and rodent RasGRP1 or 3 [6,10]. Anti-pan-Ras (Ab-4) was from Oncogene Science. Anti-H-Ras, K-Ras, N-Ras, anti-HB-EGF C-terminal domain were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Y13-259 rat monoclonal anti-Ras antibody was purified from the supernatant of hybridoma cells (ATCC, Manassas, USA). 4G10 anti-phosphotyrosine, polyclonal anti-Shc, anti-EGFR, anti-Grb2 and horseradish peroxidase (HP)-conjugated rabbit anti-sheep-IgG were from Upstate (Charlottesville, USA). Anti-HB-EGF neutralizing antibody directed against the extracellular, secreted portion was from R&D Systems (Minneapolis, USA). Anti-Phospho-p44/42-Erk(T402/Y404) and anti-Phospho-Akt(T308) were from Cell Signaling (Beverly, USA). Anti-pan-p44/42-Erk, monoclonal anti-Shc and anti-Sos-antibody were from BD-Transduction Laboratories (San Jose, USA). HP-conjugated rabbit anti-mouse IgG was obtained from KPL (Gaithersburg, USA).

### Cell culture

COS-7 cells (ATCC, Manassas, USA) were grown in DMEM supplemented with 10 % fetal calf serum at 37°C in a 5 % CO<sub>2</sub> incubator. For all experiments cells were serum-starved 24 hr in DMEM.

### Ras-GTP pull-down assay

Serum-starved COS-7 cells were treated/stimulated as appropriate and lysed in 1 ml (6-well plate) or 1.5 ml (10 cm dish, for up-scaling and visualization of basal Ras-GTP levels) of ice cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 5 mM MgCl<sub>2</sub>, 1 % NP-40, protease inhibitors, 100  $\mu$ M GDP) supplemented with 20  $\mu$ g/ml soluble GST-Raf-1-RBD (produced in *E.coli* by standard procedures). Cell material was scraped off and lysates were cleared by centrifugation. GST-Raf-1-RBD/Ras-GTP complexes were collected on Glutathione-Sepharose, washed twice with 250  $\mu$ l lysis buffer lacking GDP and GST-Raf-1-RBD, followed by electrophoretic separation of the washed precipitates and Western blot analysis with pan-Ras antibody alone (6-well plates) or in combination with K-Ras, H-Ras and N-Ras antibodies (10 cm dishes).

### Permeabilization, nucleotide exchange measurement and TLC analysis of Ras-bound nucleotides

COS-7 cell permeabilization was performed essentially as described previously [28]. Serum-starved sub-confluent COS-7 cells in 6-well plates were incubated for at least 10 min in (50 mM HEPES pH 7.5, 107 mM potassium glutamate, 15 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.3 mM CaCl<sub>2</sub>, 1 mM EGTA) at 37°C in the presence or absence of relevant inhibitors. The medium was changed for 0.6 ml of the same solution at 37°C containing 1 unit/ml streptolysin O and 500 µM Mg-ATP. For inhibitor-treated cells, this solution was supplemented with the relevant drugs. This solution was also supplemented with 100 nM TPA for analysis of nucleotide uptake 5 min post-stimulation. 4 min after this addition 100 µl of the same solution containing 400-500 µCi/ml [ $\alpha$ -<sup>32</sup>P]GTP was added to each well. This solution was supplemented with 100 nM TPA for analysis of nucleotide uptake 1 min post-stimulation (if not indicated otherwise, TPA stimulation was always 5 min prior to starting analysis of nucleotide uptake). Kinetics were started 1 min later (i.e. 5 min after addition of streptolysin O): medium was removed and cells were lysed in 1 ml ice cold (50 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 % NP-40, 100 µM GDP, 100 µM GTP, protease inhibitors) supplemented with 5 µg/ml Y13-259 antibody for Ras immunoprecipitation. Cells were scraped off and extracts were put on ice. Lysates were cleared by centrifugation and supernatants were made up to 500 mM NaCl, 0.5 % sodium deoxycholate, 0.05 % sodium lauryl sulphate. Immunocomplexes were collected on GammaBind-Sepharose by 45 min incubation in the cold. After six rounds of washing with 1 ml ice cold buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 % Triton X-100, 0.005 % SDS) immunoprecipitates were sequentially subjected to Cerenkov counting and TLC analysis exactly as described by others [1]. In all panels time point 0 as recorded is defined by quenching of the first assay point 5 min after streptolysin O addition.

### Immunoprecipitation and Western blotting

For EGFR and Shc immunoprecipitation experiments, cells were treated as appropriate and lysed in (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, protease inhibitors, phosphatase inhibitors). Alternatively, in those cases where immunoprecipitation was combined with Ras-GTP pulldown assays, immunoprecipitations were performed on the supernatant of the Ras-GTP pulldown once GST-Raf-1-RBD had been collected on the glutathione-sepharose. In either case cell extracts were split and incubated for 2 hr with 1 µg of immunoprecipitating antibody for EGFR (sheep polyclonal) or Shc (rabbit polyclonal). Immunocomplexes were collected on GammaBind-Sepharose by an overnight incubation. Immunoprecipitates were washed three times with lysis buffer and resolved by SDS-PAGE. For immunoblotting, membranes were blocked and probed with

4G10 mouse anti-phosphotyrosine and mouse anti-Grb2. Blots were then incubated with the appropriate secondary antibodies, all conjugated with horseradish peroxidase and the immunoreactive bands were visualized by enhanced chemoluminescence. PVDF-membranes probed with 4G10 anti-phosphotyrosine were stripped and reprobed with sheep anti-EGFR and mouse monoclonal anti-Shc.

#### Analysis of HB-EGF shedding

Serum-starved COS7-cells ( $2-3 \times 10^6$  cells) were treated with 100 nM TPA for the indicated time periods. For detection of secreted HB-EGF, the culture medium was supplemented with 200  $\mu$ M PMSF, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin and incubated for 2 hr with heparin-agarose beads in the cold. The heparin-agarose precipitates were washed once with ice-cold buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 5 mM  $MgCl_2$ , 1 % NP-40, protease inhibitors), eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE and Western blotting with goat anti-HB-EGF neutralizing antibody. For detection of proHB-EGF, cells were lysed in 500  $\mu$ l of lysis buffer. Lysates were clarified by centrifugation and aliquots were subjected to SDS-PAGE and Western Blot analysis with goat anti-proHB-EGF directed against the C-terminal domain of proHB-EGF.

#### Reversible oxidation of PTPs

PTP oxidation was assayed by the method of Meng et al. [29]. Briefly, serum-starved COS7-cells were challenged with 100 nM TPA or hydrogen peroxide for the indicated time periods. To prevent oxidation of the catalytic cysteine in Protein-Tyrosine-Phosphatases (PTP), cells were carefully lysed in 150  $\mu$ l of lysis buffer (25 mM sodium-acetate, pH 5.5, 150 mM NaCl, 1% NP40, 10% glycerol, protease inhibitors) that had previously been vacuum-degassed for 4 h. Lysis was carried out in the presence or absence of 10 mM iodoacetic acid. Lysates were incubated for 30 minutes on ice to ensure complete alkylation and cleared by centrifugation at 14000 rpm, 4°C for 15 minutes. Aliquots were then subjected to a PTP in-gel assay exactly as described [29]. SDS-polyacrylamide gels were cast by standard procedures except that  $^{32}P$ -labeled poly(Glu<sub>4</sub>Tyr) was added to the polymerization mix at  $10^4$  Bq/ml gel solution. Gels were dried and exposed to a phospho-imager.

#### Northern blotting

Northern blotting for detection of RasGRP1 and RasGRP3 mRNA was performed by routine methodologies. In brief, total RNA of COS7, Jurkat T-cells, Ramos B-cells and cerebral cortex of the green monkey (*Cercopithecus aethiops*) (a kind gift of Dr. W. Enard, Leipzig) was isolated using QIAamp RNA Mini Kit (Qiagen, Hilden, Germany), separated by denaturing electrophoresis,



vacuum-transferred to a nylon membrane and hybridized with [ $\alpha$ - $^{32}$ P]dATP-labelled DNA probes derived from human RasGRP1 (encompassing bp 1-918; an alternative probe encompassing bp 918-2052 was used in a separate experiment yielding the same results as shown in Figure 8B) or human RasGRP3 (encompassing bp 1-1187). Labelling was done by random priming (Roche Molecular Biochemicals, Mannheim, Germany) and hybridization was performed under stringent conditions. After exposure, membranes were stripped and reprobed for  $\beta$ -actin.

## Results

Phorbol ester stimulates nucleotide exchange on Ras. The Phorbol ester Phorbol-12-myristate-13-acetate (TPA) induces sustained and robust Ras activation in COS-7 cells (Figure 1A) in a dose-dependent manner (Figure 1B) [5]. We chose this cell type for the current study of PE-induced Ras activation because various mechanisms have been documented or inferred to operate in COS-7 cells: 1) PE-induced Ras activation is sensitive to PKC inhibitors [5], 2) PE transactivates the EGFR leading to tyrosine phosphorylation of Shc [15,18,21], 3) PE has been inferred to activate Ras through direct activation of RasGRP1 [30] and finally, 4) Ras and Erk activation in response to PE are not compromised by S17N-Ras in COS-7 cells [5,8,9], which has been interpreted by some investigators as an indication for PE-induced GAP down-regulation [4].

To systematically address the mechanism of TPA-induced Ras activation, we first designed experiments that would discriminate between GEF activation and GAP inhibition. To this end we employed a cell permeabilization strategy that allows for the rapid delivery of radioactively labeled guanine nucleotides into cells. Quantification of radioactivity uptake by Ras over time provides information on the nucleotide exchange rate by Ras proteins. Nucleotide uptake monitored by this assay is specific, since it is ablated by omission of the immunoprecipitating antibody Y13-259 or the permeabilizing agent streptolysin O, and reversible, since it is chased by recombinant Ras or unlabelled GTP but not adenine or xanthine nucleotides (Figure 2A and data not shown) [31]. Importantly, the Y13-259 antibody precipitated quantitatively K-, H-, and N-Ras and, contrary to previous observations [32], it did not cross-react with endogenous TC21 or M-Ras proteins (data not shown). To evaluate whether or not PE stimulated nucleotide exchange on Ras, serum-starved COS-7 cells were challenged with TPA 1 min or 5 min prior to permeabilization and quenching of the first assay point. As shown in Figure 2B, TPA elevated the rate of nucleotide uptake by Ras. Stimulation of nucleotide uptake was more pronounced in cells challenged 5 min versus 1 min prior to pore formation, illustrating that stimulation of nucleotide exchange increased with time (quantified in Figure 2C). Increased uptake of guanine nucleotides in response to PE was accompanied by a percentage accumulation of Ras-GTP in the same immunoprecipitates (Figure 2D), strongly indicating that the PE-induced increase in nucleotide uptake was not an incidental event but was causally linked to the activation of Ras. Of note, initial values for % Ras-GTP/(GDP + GTP) started off high and leveled off only at later time points. This pattern most probably reflected the different time required for single Ras proteins to reach steady-state nucleotide turnover versus that needed for the whole Ras population to run through one round of nucleotide exchange. Taking all these data together, we conclude that TPA activates Ras-GEF(s) to stimulate nucleotide exchange on Ras.

Transactivation of the EGFR/Shc pathway does not mediate Ras activation by TPA. TPA elicits sequential tyrosine phosphorylation of the EGFR and the adapter protein Shc in COS cells [15,18]. Since it is well established that this pathway feeds into the Ras-GEF Sos, it was a likely mediator of TPA-induced nucleotide exchange stimulation. To address this possibility we used a pharmacological approach to inhibit EGFR activity. The well-characterized selective EGFR-inhibitor AG1478 completely ablated TPA-induced EGFR and Shc phosphorylation (Figure 3), in agreement with a previous report [21]. AG1478 treatment also compromised basal levels of EGFR and Shc phosphorylation. By contrast, AG1478 caused only a partial attenuation of Ras-GTP accumulation in response to TPA (Figure 3, see quantification below) indicating that the bulk of Ras-GTP formation following TPA stimulation did not require the EGFR. Assessing the activation status of the Ras effector Erk-pathway yielded an analogous picture, that is, application of AG1478 caused inhibition of basal Erk activity but only minor mitigation of TPA-sparked Erk activation.

To investigate the role of PKC, a major target of TPA, we tested the effect of the PKC inhibitor bisindolylmaleimide I (BIM), which reportedly blocks TPA activation of Ras/Erk in COS cells [5] (Figure 3). In accordance with this and other reports [16,21], 500 nM BIM treatment strongly or fully impaired TPA-induced Ras-GTP accumulation, EGFR transactivation, tyrosine phosphorylation of Shc and Erk activation, suggesting that TPA signaled through PKC both to the EGFR/Shc and into the Ras/Erk pathway. Importantly, basal activation/phosphorylation levels of all parameters were not affected by BIM. In summary, while EGFR inhibition compromised basal levels of the Ras/Erk pathway but did not significantly impinge on TPA-induced Ras/Erk activation, PKC inhibition specifically interrupted TPA-dependent effects but not basal levels of activation. These data strongly indicated that PKCs acted as genuine mediators of TPA-elicited signalling while EGFR activity sustained the steady-state levels of the Ras/Erk-pathway. In addition, these observations illustrated that the EGFR/Shc and Ras/Erk pathways were engaged in parallel rather than in series downstream of TPA-activated PKC.

EGFR sustains basal Ras-GTP levels in serum-arrested cells. Figure 3 illustrates that EGFR inhibition with AG1478 compromised basal tyrosine phosphorylation of Shc, suggesting that the interruption of basal Erk-pathway activation occurred upstream of Ras. To address this hypothesis, we first investigated complex formation between Shc and Grb2 adapter proteins, a commonly used readout for the engagement of the Grb2/Sos GEF system (Figure 4A). TPA-stimulated as well as basal Shc/Grb2 association were ablated upon AG1478 treatment, indicating that inhibition of basal

EGFR activity disrupted the Shc/Grb2/Sos GEF system in serum-arrested cells. To investigate whether disruption of the Shc/Grb2 complex formation impinged on basal Ras-GTP levels, we scaled up the Ras-GTP pulldown assay in order to detect basal levels of Ras-GTP (Figure 4B) (see experimental section). AG1478 treatment resulted in a marked reduction of basal Ras-GTP levels, confirming that basal EGFR activity drove steady-state Ras-GTP formation. The structurally related tyrphostine AG1296, a selective inhibitor of the PDGFR (which is not expressed in COS-7 cells), had no effect on basal Ras-GTP levels (data not shown). As shown above, TPA raised Ras-GTP levels in the background of AG1478 inhibition (Figure 4B). While Ras-GTP accumulation did not reach the level obtained from untreated cells, densitometric analysis of Ras-GTP signals and correction for the basal Ras-GTP values prior to TPA administration revealed that net accumulation of Ras-GTP in response to TPA was largely unaffected by AG1478 (Figure 4G). The same pattern was observed at the level of Erk (Figure 4B).

To underscore this finding with an alternative approach, we employed the diphtheria toxin mutant CRM197, which interrupts TPA-induced EGFR transactivation in COS-7 cells by scavenging HB-EGF [15]. In a preliminary experiment we assessed whether or not TPA induced the processing of endogenous HB-EGF in COS-7 cells, since in this cell type, this process has as yet only been documented in cells engineered to overexpress HB-EGF [15,33]. Cells were stimulated with TPA for various time frames and heparin-binding molecules released into the medium were collected by affinity purification on heparin-agarose. Immunoblotting with an antibody that targets the extracellular, secreted portion of HB-EGF (sHB-EGF) revealed time-dependent proHB-EGF processing which was detectable 5 to 7 min after TPA administration (Figure 4C and D). Immunoblotting of the corresponding cell extracts with serum directed against the intracellular domain revealed a heterogeneous pattern of proHB-EGF polypeptides, as reported previously by others [15,34]. The observed shedding of HB-EGF suggested that sHB-EGF was indeed a potential mediator of TPA-elicited EGFR transactivation. In line with this notion and previous reports [33,34] TPA-induced processing of HB-EGF was highly sensitive to PKC inhibition (Figure 4D). A role for HB-EGF shedding in TPA-induced transactivation of the EGFR was further corroborated by the finding that sequestration of HB-EGF by CRM197 blocked EGFR transactivation in response to TPA (Figure 4E) [15]. However, CRM197 application resulted in only weak attenuation of TPA-induced Ras-GTP formation (Figure 4E and G) or Erk activation (data not shown), in line with previous observations made at the level of Erk [22]. These results confirmed that the EGFR did not transduce TPA signals to Ras, while it was critical for the maintenance of basal Ras-GTP levels.

In marked contrast, the effects of the PKC inhibitor BIM were the mirror image of those elicited by EGFR inhibition, that is, BIM did not affect basal Ras-GTP levels but strongly compromised TPA-induced Ras-GTP accumulation (Figure 4F,G). In Figure 4G, Ras-GTP levels are plotted both in terms of absolute values and as fold of basal to highlight the fundamentally opposed consequences of EGFR versus PKC inhibition. Taken together, these data demonstrated that PKC and EGFR independently regulated TPA-induced and basal Ras-GTP formation, respectively.

Two independent nucleotide exchange factor systems regulate basal and TPA-driven nucleotide exchange on Ras. Since Ras activation by TPA proceeds via stimulation of GEFs (Figure 2B,C), we went on to determine the consequences of EGFR or PKC inhibition at the level of nucleotide exchange on Ras. The aim of these experiments was to determine, whether the observed role of PKC and EGFR in TPA-induced versus basal Ras-GTP formation reflected the regulation of different Ras-GEF systems by PKC and EGFR. COS-7 cells were treated with AG1478 to inhibit the EGFR prior to TPA administration and permeabilization in the presence of radiolabeled GTP (Figure 5A). EGFR inhibition resulted in a drop of the basal nucleotide exchange rate on Ras. Quantification revealed a reduction of overall basal nucleotide exchange by about 40 % following EGFR inhibition (Figure 5E). However, since these values include the intrinsic nucleotide exchange rate of Ras proteins (the exact magnitude of which is arduous to estimate), the contribution of the EGFR to GEF-catalyzed nucleotide exchange in resting COS-7 cells is bound to exceed 40 %. This observation demonstrated that basal EGFR activity sustained steady-state Ras-GTP levels via stimulation of nucleotide exchange in serum-starved cells. Strikingly, the breakdown of basal nucleotide exchange caused by EGFR inhibition did not compromise TPA signalling to GEFs since TPA was able to mount a response and raise nucleotide uptake in the presence of AG1478 (Figure 5A; quantified in Figure 5E). Remarkably, fold-stimulation of overall nucleotide exchange by TPA was even increased in a background of EGFR inhibition (Figure 5E; see discussion). Separation of guanine nucleotides associated to the same immunoprecipitates showed that TPA-induced accumulation of Ras-GTP was not compromised by the breakdown of basal nucleotide exchange elicited by AG1478 (Figure 5B), confirming the results of the Ras-GTP pulldown experiments shown above. Since EGFR and Shc are not phosphorylated/activated under these conditions (Figure 3 and 4A), the straightforward interpretation is that TPA addresses a Ras-GEF system different from the canonical Shc/Grb2/Sos module that acts downstream of the EGFR.

In analogous experiments, the consequences of PKC inhibition with BIM were again the mirror image of AG1478 effects: BIM did not compromise basal nucleotide exchange on Ras but it

markedly impaired stimulation of nucleotide uptake and accumulation of GTP-loaded Ras in response to TPA (Figure 5C,D; quantified in Figure 5E), strongly supporting the idea that PKC is a genuine constituent of the TPA signalling pathway that feeds into Ras-GEF(s).

Reactive oxygen species mediate EGFR transactivation by TPA. The experimental data presented so far indicated that TPA signals bifurcated downstream of PKC leading to the parallel activation of the EGFR and the Ras/Erk pathway. This raised the question as to the molecular mechanism underlying this splitting of signals downstream of PKC. Several reports have documented that TPA potently induces reactive oxygen species (ROS) via PKC in a number of cell types [35,36]. An increasing body of evidence implicates reversible inhibition of protein tyrosine phosphatases (PTP) as one major mode of action of ROS in growth factor and PE signalling [35,36]. Reversible PTP inhibition results from the oxidation of the catalytic cysteine to sulphenic acid by ROS. To explore the involvement of ROS and ROS-dependent PTP down-regulation in TPA signalling to the EGFR or the Ras/Erk pathway, we investigated PTP inhibition using a recently developed technique that monitors the reversible oxidation/inhibition of PTPs [29]. Cell extracts of TPA-stimulated COS-7 cells were prepared in the presence of iodoacetic acid, which irreversibly alkylates the catalytic cysteine of virgin, non-oxidized PTPs, but not that of their oxidized counterparts. Extracts were separated by SDS-PAGE, followed by in-gel renaturation of PTPs and an in-gel phosphatase assay. This procedure therefore only scores the phosphatase activity of reversibly oxidized PTPs, since these species are protected against irreversible, post-lytic alkylation by iodoacetic acid. As shown in Figure 6A, TPA induced the reversible oxidation/inhibition of several PTP species in a time-dependency that matched both EGFR transactivation and Ras-GTP accumulation (Figure 3). To discern whether ROS-dependent processes were involved in activation of either of the two pathways, cells were treated with the thiol-group containing reducing compound N-acetyl-cysteine (NAC) prior to TPA administration (Figure 6B). NAC almost completely abrogated TPA-induced EGFR transactivation. By contrast, Ras-GTP formation and Erk activation in response to TPA were not compromised by NAC treatment. Moreover, direct application of hydrogen peroxide resulted in tyrosine phosphorylation of the EGFR but it did not promote Ras-GTP formation or Erk activation indicating that COS-7 cells generally lacked the ability to couple ROS to Ras activation. Taken together, these observations identified ROS as a link between PKC and EGFR transactivation and underscored the conclusion that EGFR transactivation and activation of the Ras/Erk pathway proceeded via separate pathways downstream of TPA-activated PKC (Figure 10).

EGFR mediates PI3K/Akt activation by phorbol ester. Since the EGFR became transactivated by TPA but did not transduce the signal for Ras activation, we addressed the question whether PE-induced EGFR activation mediated PE effects other than Ras activation. As a second major signalling pathway we investigated Akt activation by PE. PE elicited the time-dependent activation of Akt, as monitored by phosphorylation of Ser473 (Figure 7). PE-induced Akt activation was fully absent from AG1478-treated cells, showing that EGFR transactivation mediated Akt activation induced by PE. Hence, while EGFR transactivation by PE was unproductive in terms of Ras activation, it did mediate Akt activation in response to PE.

COS-7 cells do not express RasGRP1 or RasGRP3. The current data illustrated that PE-induced nucleotide exchange on Ras and concomitant accumulation of Ras-GTP proceeded in the absence of tyrosine phosphorylation of Shc and Shc/Grb2 association. This observation excluded the canonical Shc/Grb2/Sos system as the GEF unit addressed by PE. Two other classes of Ras GEFs, RasGRF and RasGRP, could potentially mediate PE activation of Ras. RasGRP proteins are particularly attractive since they interact physically with TPA and mediate TPA/DAG-induced Ras activation [6,10-14]. Recent work by Bivona et al. has implicated RasGRP1 activity in DAG and TPA-dependent signalling in COS cells by use of two dominant-negative RasGRP versions [30]. However, since endogenous expression of RasGRP1 was not demonstrated in that study, we investigated RasGRP1 expression in COS-7 cells by Northern blotting (Figure 8). In parallel we also assessed expression of the related GEF RasGRP3, because RasGRP3 shows the widest tissue distribution among RasGRP isoforms (see discussion). To ascertain the functionality of the hybridization probes we included two types of controls: firstly, we included total RNA from Jurkat T-cells and Ramos B-cells in this analysis, since these cell lines express RasGRP3 (Ramos) and RasGRP1 (Jurkat and, to a lesser extent, Ramos) [6,10,12]. Secondly, since COS-7 cells originate from green monkey, we prepared total RNA from the brain cortex of green monkey and included this sample in the experiment (see Figure 8A for total load of RNA). Various transcripts for RasGRP1 around 4 Kb in size were detected in Jurkat and, with lower intensity, also in Ramos cells and green monkey brain (Figure 8B). The latter observation demonstrated that the hybridization probe was suited for the detection of monkey RasGRP1 mRNA, particularly if one considered that total RNA load was 3-fold less for the green monkey brain sample (see legend to Figure 8A). A transcript for RasGRP3 of about the same size was detected only in Ramos cells (Figure 8B). As regards the two leukocyte cell lines, the pattern obtained here is virtually identical to previous data both in terms of the transcript size and the differential expression of RasGRP1 and 3 in the two cell types [6,10,12,14]. Importantly, neither RasGRP1 nor RasGRP3 message was detected in COS-7

cells, indicating that neither GEF is expressed in this cell line. However, this conclusion was not straightforward in the case of RasGRP3, since a RasGRP3 transcript could not be detected in green monkey brain. Since this observation raised the possibility that the Northern probe for human RasGRP3 did not hybridize to monkey mRNA, we went on to assess RasGRP1 and 3 expression in COS-7 cells at another level. COS-7, Jurkat and Ramos cell extracts were subjected to Western blotting using isoform-specific polyclonal antibodies (Figure 8C). Both antibodies have previously been shown to react with human and rodent RasGRP1/3 proteins, respectively [6,10], indicating that they should readily recognize the monkey orthologs. As shown in Figure 8C, the result of the Western blot was essentially the same as that obtained in the Northern experiment. Importantly, RasGRP1 and RasGRP3 were not detectable in COS-7 cell lysates. A commercially available antibody against RasGRP1 (Santa Cruz Biotechnology, H-120) which recognizes human and mouse RasGRP1 did also not detect RasGRP1 in COS-7 cells (data not shown). Taken together, these data strongly argued against the presence of RasGRP1 or RasGRP3 in COS-7 cells.

Phorbol ester induces PKC-dependent phosphorylation of Sos. Since RasGRP1 and 3 are absent from COS-7 cells and RasGRF1/2 proteins are similarly not expressed in this cell line [37], COS-7 cells are inferred to express only Sos among characterized Ras-GEF species. The most probable scenario involved an engagement of Sos by TPA-activated PKC, which did not include tyrosine phosphorylation of Shc or Shc/Grb2 complex formation. Sos might conceivably be recruited to another adapter protein-containing complex in response to TPA. Alternatively, Sos activity might change as a result of phosphorylation events. To test this latter possibility, we stimulated COS-7 cells with PE and monitored changes in electrophoretic mobility of Sos, a reliable surrogate marker of Sos-phosphorylation [38-41]. As shown in Figure 9A, PE elicited a shift in Sos mobility, indicative of one or more phosphorylation events on Sos, that coincided temporally with the formation of Ras-GTP. To further characterize this phosphorylation event, we compared the effects of PE and EGF (Figure 9B). In agreement with previous findings obtained in COS-7 cells, EGF also induced a mobility shift of Sos [38], albeit of lower intensity than the one induced by TPA. The EGF-induced shift was blocked by AG1478 or the MEK inhibitor PD98059, indicating that Sos-phosphorylation occurred downstream of Ras via MEK-dependent feedback loops, as reported previously [38-41]. By contrast, the TPA-induced shift in Sos mobility was not affected by AG1478 treatment and was only partially sensitive to MEK inhibition, whereas PKC inhibition with BIM fully ablated the PE-induced shift. Taken together, these data indicated that Sos became phosphorylated in response to PE in a PKC-dependent fashion upstream of Ras.



## Discussion

Several mechanisms for Ras activation by Phorbol esters have been reported in past studies. In peripheral T-cells, PKC-dependent inhibition of GAP proteins was originally put forward as the major pathway, as concluded from the observation that TPA did not increase nucleotide turnover on Ras [1]. By contrast, more recent reports have presented solid genetic and biochemical evidence arguing for a critical role of the GEF RasGRP1, implying that PE-induced Ras activation in T-cells proceeds via stimulation of nucleotide exchange [10-13]. Working on COS-7 cells we demonstrate that TPA stimulates nucleotide exchange on Ras, strongly indicating that TPA targets Ras-GEF(s) in these cells. Previous studies have illustrated that TPA stimulates nucleotide exchange on Ras in permeabilized cardiac myocytes [2] and pancreatic acinar cells [42], indicating that GEF engagement may be the more widespread mechanism underlying TPA activation of Ras. With regard to the situation in T-cells, the reported absence of nucleotide exchange stimulation and the increasing body of evidence arguing for RasGRP1 engagement by TPA are difficult to harmonize. One possible way of reconciling both lines of evidence relates to the particular features of Ras in those cells. Leukocytes exhibit a very high basal rate of GDP/GTP cycling on Ras [1,43], implying that a small fractional increase in nucleotide exchange could result in a large accumulation of Ras-GTP owing to signal amplification provided by the high Ras-GDP/GTP cycling rate. Based on our experience with the permeabilization methodology, we consider it possible that a modest increase in nucleotide exchange, as conceivably mediated by RasGRP1, might have passed undetected in the relevant experiments. However, neither these considerations nor the experimental data presented here can rigorously rule out down-regulation of GAP activity by TPA, the more so since TPA stimulation reportedly reduces the amount of GAP activity that is recovered from T-cell extracts [1].

In COS-7 epithelial cells, TPA transactivates the EGFR via PKC and it has been proposed that this crosstalk culminates in Ras activation via the canonical Shc/Grb2/Sos GEF system [15,18,21]. However, while this pathway is blocked by dominant-negative S17NRas in COS-7 cells challenged with EGF, S17NRas does not affect Ras and Erk activation upon TPA stimulation of these cells [5,8,9]. These data indicated that a model, in which TPA usurps the EGFR/Shc/Grb2/Sos pathway was unlikely to account for the Ras activation mechanism in COS-7 cells. The findings presented here are in line with these theoretical considerations, since they demonstrate that the EGFR is not an integral constituent of the signalling pathway linking TPA to Ras/Erk. Three experimental manipulations uncouple TPA-induced EGFR transactivation from Ras-GTP formation, and hence illustrate that EGFR transactivation does not mediate Ras activation by TPA: 1. inhibition of the EGFR with the selective inhibitor AG1478, 2. sequestration of HB-EGF with the diphtheria toxin

mutant CRM197, and 3. interference with ROS generation by the reducing agent NAC. In all three cases, Ras-GTP accumulation proceeds almost unaffected upon addition of TPA in the absence of EGFR transactivation or even in a background of complete EGFR inhibition. Moreover, EGFR inhibition with AG1478 did not compromise the up-regulation of nucleotide exchange induced by TPA, demonstrating unambiguously that engagement of RasGEFs by TPA is not mediated by the EGFR. Since TPA-induced tyrosine phosphorylation of Shc and Shc/Grb2 complex formation are totally dependent on EGFR activity (Figures 3 and 4A) [15], it follows that TPA targets a RasGEF system different from the canonical Shc/Grb2/Sos pathway.

It is noteworthy that previous investigations on the role of the EGFR in TPA signalling to the Ras downstream effector Erk in COS-7 cells had yielded seemingly discrepant results. Roudabush et al. reported that activation of endogenous Erk by TPA was unaffected by AG1478 or CRM197 [22], in agreement with the observations made here both at the level of Erk and Ras. By contrast, two laboratories have documented about 50 % reduction in TPA-elicited Erk activation by AG1478 in COS-7 cells [18,21], leading to the conclusion that EGFR transactivation relays part of the TPA signal to the Ras/Erk pathway. It should be noted that one of the latter studies investigated activation of ectopically expressed Erk [18], while in the other a supra-optimal concentration of AG1478 was used (5  $\mu$ M) [21], casting some doubts on the specificity of the reported effects. This notwithstanding, our finding that the EGFR maintains basal levels of Erk activation (which, as noticed previously by others [38], are rather high in COS cells compared to other commonly investigated fibroblast and epithelial cell lines) provides a plausible explanation for these divergent observations. As illustrated in Figure 3, Erk activation in response to TPA starts off from almost zero basal Erk activity levels in AG1478-treated cells, implying that Erk activity induced by TPA will not reach the value obtained in virgin cells. Indeed, in analogy to the data for Ras-GTP accumulation (Figure 4G), we find that maximum Erk phosphorylation upon TPA stimulation of AG1478-treated cells generally amounts to 80-90 % of the value from AG1478-untreated cells. The current study highlights that this gap in Erk activity is attributable to the loss of basal Erk phosphorylation that occurs upon inhibition of basal EGFR activity, rather than to a defect in TPA signalling. This effect of AG1478 may be exacerbated by clonal differences among COS-7 batches affecting the relative magnitude of basal (AG1478-sensitive) versus TPA-responsive (AG1478-insensitive) Ras and Erk activities. In conclusion, we propose that a number of apparent discrepancies regarding the role of EGFR transactivation in Ras/Erk activation by TPA (and possibly also other agonists) can be rationalized by taking into consideration the contribution of basal levels of activation of the Ras/Erk pathway.

EGFR transactivation by heterologous extracellular agonists is a widespread phenomenon. A large body of evidence demonstrates that EGFR inhibition compromises Erk activation by numerous stimuli in a number of cell types, including COS-7 cells, arguing for the EGFR as a point of convergence for many agonists that signal to the Ras/Erk pathway. A previous study on the role of the EGFR in lysophosphatidic acid (LPA)-induced Ras activation has shown that EGFR inhibition impairs the net accumulation of Ras-GTP upon LPA stimulation of COS-7 cells [28], in apparent support of the mentioned scenario. However, EGFR inhibition did not prevent LPA-induced stimulation of nucleotide exchange [28]. This paradoxical effect of EGFR inhibition – block of LPA-induced Ras-GTP formation but no interference with nucleotide exchange stimulation - reflected a marked drop in the rate of basal nucleotide exchange following EGFR inhibition, which could not be fully compensated by LPA despite unimpaired stimulation of nucleotide exchange. The same phenomenon, i.e. a decline in basal activity levels of the Ras/Erk pathway following EGFR inhibition is also documented in the present report at the level of Ras-GTP formation (Figure 4B,G), Ras nucleotide exchange (Figure 5A,E), and Erk activity (Figure 3 and 4B), and was previously illustrated by others at the level of Erk activation [22]. Taken together, all these observations indicated that it is the maintenance of basal nucleotide exchange on Ras by basal EGFR activity that is critical to the action of LPA. With regard to the present discussion, these considerations lend strong support to the notion that transactivation of the EGFR does not mediate Ras/Erk pathway activation by TPA in COS-7 cells. This conclusion is further reinforced by the converse observation, namely the fact that TPA induces pronounced tyrosine phosphorylation of the EGFR and/or Shc in cell types such as HEK-293 [15] or Rat-1 fibroblasts [19], in which TPA fails to activate Ras [31,44].

The finding that the EGFR drives the formation of basal Ras-GTP levels but not TPA or LPA-induced Ras-GTP accumulation raises a further question: if EGFR inhibition does not compromise nucleotide exchange stimulation in response to LPA [28] or TPA (this report) why does AG1478 eradicate Ras-GTP formation only in the former case [28]? Two explanations can account for this observation: firstly, TPA but not LPA may down-regulate GAP activity in addition to stimulating GEFs. In consequence, TPA-induced GEF stimulation and GAP inhibition would amplify each other's effect and give rise to the full response of Ras-GTP formation that is observed throughout this study. By contrast, if LPA action only relies on GEF stimulation, this effect may be strong enough to redress the balance of nucleotide exchange following its breakdown upon EGFR inhibition, as illustrated in reference 28, but it may not be potent enough to result in net Ras-GTP

formation. Since several lines of evidence argue for GAP inactivation as a mechanism evoked by TPA [1,4], it is not unlikely that this scenario may apply to COS-7 cells.

Alternatively or additionally TPA and LPA could address different pools of Ras within COS-7 cells. An increasing body of experimental evidence indicates that Ras proteins segregate into various sub-cellular organelles as well as into distinct plasma membrane microdomains in dependence of the Ras isoform, activation status, palmitoylation status and possibly other as yet unknown cues [45]. It seems reasonable to assume that the function of the EGFR in maintaining steady-state nucleotide exchange on Ras applies to one or a subset, but not all of the various compartments. In consequence, the breakdown of basal Ras nucleotide exchange upon EGFR inhibition would impinge on agonists that target the same Ras population (hypothetically: LPA), whereas Ras activation by cues that feed into a different Ras pool(s) (hypothetically: TPA) would not be affected. A particular appeal of this scenario resides in that, additionally, it provides a rational explanation for the distinct sensitivity of TPA versus EGF and LPA signalling towards S17NRas: if S17N Ras segregates exclusively into the EGF and LPA responsive Ras pool (following the line of reasoning started above), it should not affect nucleotide exchange in a separate Ras pool that is addressed by TPA. Circumstantial evidence in support of the existence of various Ras populations that are distinctly addressed by agonists is provided by the data shown in Figures 4G and 5E, according to which the TPA-induced fold-increase in nucleotide exchange and Ras-GTP formation is enhanced in a background of EGFR inhibition. The easiest interpretation of this surprising observation is that an EGFR-driven component of basal nucleotide exchange in a TPA-unresponsive Ras population had obscured the real magnitude of the TPA effect. In other words, the loss of basal nucleotide exchange upon AG1478 application reduces the overall basal rate without affecting TPA action, hence resulting in an apparent higher fold-stimulation of exchange and Ras-GTP formation.

The current data hence argue for the independent activation of the Ras/Erk and EGFR/Shc pathways downstream of TPA-activated PKC in COS-7 cells (Figure 10). While TPA induces EGFR transactivation via PKC-dependent shedding of HB-EGF, this pathway does not mediate activation of Ras, but is responsible for activation of PI3K and Akt (Figure 7) and possibly other pathways. It is worth raising the point (previously noticed by others [46]) that the kinetics of TPA-induced HB-EGF processing, as illustrated here in Figure 4C, at first sight appears to be in conflict with the time course of EGFR transactivation (e.g. Figure 3) and EGFR-dependent Akt phosphorylation (Figure 7), since both latter processes are switched on before HB-EGF release into the medium becomes detectable. However, HB-EGF processing is likely to proceed with faster kinetics than those

illustrated in Figure 4C, since the immediate release of shed HB-EGF to the medium is prevented by its association with heparan sulphate proteoglycans on the cell surface [15,47]. Indeed, initial studies on the mechanism of EGFR transactivation failed to detect soluble EGF ligands in the medium at all [17,48], and it was only upon more careful analysis, that processing of HB-EGF and other latent membrane-bound EGF-like precursor proteins were detected and implicated in the process of EGFR transactivation [15,33,34]. In conclusion, as proposed previously by other investigators [15], these considerations suggest that the delayed kinetics of HB-EGF shedding as illustrated in the present study must not be at odds with a role for HB-EGF as a mediator of agonist-induced EGFR transactivation.

An unsolved issue regards the identity of the RasGEF system(s) that is activated by TPA via PKC. The data presented here suggest that TPA does not address the canonical Shc/Grb2/Sos system. Moreover RasGRF1 and RasGRF2 are both not expressed in COS cells [37]. On the other hand, members of the RasGRP family of RasGEFs are potential candidates, since they are direct target proteins of TPA/DAG. We focused our interest on the two family members RasGRP1 and RasGRP3 for a number of reasons: 1) RasGRP1 and RasGRP3 are the two species most solidly established to function as mediators of TPA-driven Ras activation, 2) RasGRP1 and RasGRP3 are activated both by direct binding to TPA and indirectly via phosphorylation by PKC [6,13], 3) RasGRP1 has been inferred (but not proven) to be present in COS cells in a recent study [30], 4) RasGRP1 and RasGRP3 are the only members reported to be expressed in tissues other than brain and blood cells: RasGRP3 expression has been documented in mesangial cells of the kidney [49], endothelial cells [50] and endocrine cells [51]; RasGRP1 expression has been detected in skin keratinocytes [52], 5) RasGRP2 exhibits selectivity for Rap, does not respond to TPA and does not efficiently activate the Ras/Erk pathway [53], 6) RasGRP4 is a mast cell restricted GEF [54]. Although these considerations rendered RasGRP1 and RasGRP3 very attractive candidates for the elusive GEF activity addressed by TPA in COS-7 cells, we have been unable to detect expression of either RasGRP variant in COS-7 cells. The failure to detect RasGRP1 and RasGRP3 in cell extracts by Western blotting was unlikely to result from a failure of the antibodies to recognize the monkey orthologs since both sera have been demonstrated to react with human and rodent RasGRP1 or RasGRP3 [6,10]. Similarly, since the hybridization probe used for detection of RasGRP1 mRNA by Northern blotting readily detected RasGRP1 transcripts from the brain cortex of green monkey (the animal species from which COS-7 cells originate), the negative result obtained for COS-7 cells unambiguously excluded RasGRP1 expression in this cell line. This degree of certainty could not be achieved in the RasGRP3 northern blot, since no transcripts were detected in the monkey brain. One interpretation of this

observation was that the Northern probe did not hybridize to monkey RasGRP3 mRNA. Alternatively, RasGRP3 expression levels in cells of the cortex might be low and thus lie below the level of detection. We favoured the second possibility since RasGRP3 expression within the brain has been mapped exclusively to oligodendroglia [49], and secondly, in agreement with the Northern blot experiment, RasGRP3 protein was not detectable in COS-7 cells by Western blotting. In summary, these findings demonstrated that COS-7 cells do not express functionally significant levels of either RasGRP1 or RasGRP3.

These considerations indicated that COS-7 cells express only Sos among characterized Ras-GEF species, implying that Sos most likely represented the GEF activity stimulated by TPA via PKC. However, the presumptive Sos activation mechanism was unconventional in that it did not involve tyrosine phosphorylation of Shc or Shc/Grb2 complex formation. We present evidence that PE induces phosphorylation of Sos in a PKC-dependent fashion and that the features of this phosphorylation event(s) are consistent with it mediating the activation of Ras by PE. Thus, in three independent experiments of the kind shown in Figure 9A, the initiation of the Sos mobility shift upon TPA administration correlated in time with the onset of Ras-GTP accumulation. PE and growth factors have previously been reported to induce phosphorylation at multiple sites within the Proline-rich carboxyl-terminal region of Sos in various cell types [38-41]. As a major hallmark shared in all referred studies, Sos phosphorylation was fully sensitive to MEK inhibition, evidencing that it resulted in all cases from Ras-sparked negative feedback loops involving Erk, Erk-dependent kinases such as Rsk-2 or other as yet unidentified MEK-dependent kinases. In agreement with these findings, we observed full MEK-dependence of Sos phosphorylation in COS-7 cells stimulated with EGF. However, in marked contrast to all reported findings, the PE-induced shift in Sos mobility was only partially reverted following MEK inhibition while it was fully absent from BIM-treated cells. It is important to note that whereas phosphorylation of some residues on Sos gives rise to reduced mobility in SDS-PAGE, other phosphorylation events do not induce changes in mobility [55]. Thus, the findings presented in Figure 9 must be considered as preliminary and interpreted with care. This notwithstanding, the most plausible explanation was that the BIM-sensitive and PD98059-insensitive phosphorylation event(s) occurred downstream of PKC and upstream of Ras whereas the PD98059-sensitive component of the mobility shift most likely reflected previously characterized MEK-dependent feedback phosphorylation of Sos. We are currently mapping the phosphorylation sites on Sos that become phosphorylated in response to PE in an attempt to clarify whether the observed PD98059-insensitive Sos phosphorylation is causally related to PE-driven Ras activation. Identification of the phosphorylation sites should also help identify the PD98059-insensitive Sos

kinase(s) engaged by PE. Since PKC $\alpha$ ,  $\beta$  and  $\gamma$  do not phosphorylate Sos *in vitro* [38], it seems more likely that PKC mediates activation of the elusive Sos-kinase in response to PE.

In conclusion, the assessment of nucleotide exchange on Ras has enabled the detection of a Ras-GEF system that is addressed by PE via PKC and independently of the EGFR. In more general terms, the present study emphasizes the relevance of basal activation levels of signalling pathway constituents and the need to include this aspect in studies aimed at deciphering the topology of signal transduction pathways. Along this line of reasoning, the confirmation of a role for the EGFR in the maintenance of basal nucleotide exchange on Ras in growth-arrested cells (Ref. 28,46 and this study) prompts the intriguing speculation that the EGFR may function to calibrate the Ras activation machinery in terms of a futile Ras-GDP/GTP cycle to regulate its responsiveness to LPA and other mitogens. Such a scenario could underlie the synergy between EGF and other mitogens that is observed in a large variety of cell types [56-59].

### **Acknowledgements**

We thank Julian Downward for helpful discussions and for sharing unpublished data. We acknowledge the generous gift of monkey brain tissue by Wolfgang Enard. We thank Frank Böhmer for critically reading the manuscript. This work was supported by “Deutsche Forschungsgemeinschaft” (DFG) grant RU860/1-1.



## References

1. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. and Cantrell, D. A. (1990) Stimulation of p21ras upon T-cell activation. *Nature* **346**, 719-723
2. Montessuit, C. and Thorburn, A. (1999) Activation of Ras by phorbol esters in cardiac myocytes. Role of guanine nucleotide exchange factors. *FEBS Letters* **460**, 57-60
3. Chiloehes, A., Paterson, H. F., Marais, R., Clerk, A., Marshall, C. J. and Sugden, P. H. (1999) Regulation of Ras.GTP loading and Ras-Raf association in neonatal rat ventricular myocytes by G protein-coupled receptor agonists and phorbol ester. Activation of the extracellular signal-regulated kinase cascade by phorbol ester is mediated by Ras. *J. Biol. Chem.* **274**, 19762-19770
4. Shu, X., Wu, W., Mosteller, R. D. and Broek, D. (2002) Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. *Mol. Cell. Biol.* **22**, 7758-7768
5. Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F. and Marshall, C. J. (1998) Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science* **280**, 109-112
6. Teixeira, C., Stang, S. L., Zheng, Y., Beswick, N. S. and Stone, J. C. (2003) Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3. *Blood* **102**, 1414-1420
7. Gschwendt, M., Kittstein, W. and Marks, F. (1993) Protein kinase C forms a complex with and phosphorylates the GTPase activating protein GAP: phosphorylation by PKC is dependent on tyrosine phosphorylation of GAP and/or a GAP-associated protein. *Biochem. Biophys. Res. Commun.* **194**, 571-576
8. Howe, L. R., Leever, S. J., Gomez, N., Nakielnny, S., Cohen, P. and Marshall, C. J. (1992) Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71**, 335-342
9. Augsten, M., Pusch, R., Biskup, C., Rennert, K., Wittig, U., Bayer, K., Blume, A., Wetzker, R., Friedrich, K. and Rubio, I. (2006) Live-cell imaging of endogenous Ras-GTP illustrates predominant Ras activation at the plasma membrane. *EMBO Rep.* **7**, 46-51
10. Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J. and Stone, J. C. (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280**, 1082-1086
11. Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L. and Stone, J. C. (2000) RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat Immunol.* **1**, 317-321
12. Coughlin, J. J., Stang, S. L., Dower, N. A. and Stone, J. C. (2005) RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J. Immunol.* **175**, 7179-7184

13. Roose, J. P., Mollenauer, M., Gupta, V. A., Stone, J. and Weiss, A. (2005) A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol. Cell. Biol.* **25**, 4426-4441
14. Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J. and Kay, R. J. (1998) Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* **18**, 6995-7008
15. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888
16. Chen, N., Ma, W.-Y., She, Q.-B., Wu, E., Liu, G., Bode, A. M. and Dong, Z. (2001) Transactivation of the epidermal growth factor receptor is involved in 12-O-tetradecanoylphorbol-13-acetate-induced signal transduction. *J. Biol. Chem.* **276**, 46722-46728
17. Tsai, W., Morielli, A. D. and Peralta, E. G. (1997) The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J.* **16**, 4597-4605
18. Grosse, R., Roelle, S., Herrlich, A., Höhn, J. and Gudermann, T. (2000) Epidermal growth factor receptor tyrosine kinase mediates Ras activation by gonadotropin-releasing hormone. *J. Biol. Chem.* **275**, 12251-12260
19. Luttrell, L. M., Daaka, Y., Della Rocca, G. J. and Lefkowitz, R. J. (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J. Biol. Chem.* **272**, 31648-31656
20. Dabrowski, A., VanderKuur, J. A., Carter-Su, C. and Williams, J. A. (1996) Cholecystokinin stimulates formation of shc-grb2 complex in rat pancreatic acinar cells through a protein kinase C-dependent mechanism. *J. Biol. Chem.* **271**, 27125-27129
21. Tebar, F., Lladó, A. and Enrich, C. (2002) Role of calmodulin in the modulation of the MAPK signalling pathway and the transactivation of epidermal growth factor receptor mediated by PKC. *FEBS Lett.* **517**, 206-210
22. Roudabush, F. L., Pierce, K. L., Maudsley, S., Dad Khan, K. and Luttrell, L. M. (2000) Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. *J. Biol. Chem.* **275**, 22583-22589
23. Piiper, A., Elez, R., You, S.-J., Kronenberger, B., Loitsch, S., Roche, S. and Zeuzem, S. (2003) Cholecystokinin stimulates extracellular signal-regulated kinase through activation of the epidermal growth factor receptor, Yes, and protein kinase C. Signal amplification at the level of Raf by activation of protein kinase Cepsilon. *J. Biol. Chem.* **278**, 7065-7072

24. Ma, C., Bower, K. A., Lin, H., Chen, G., Huang, C., Shi, X. and Luo, J. (2005) The role of epidermal growth factor receptor in ethanol-mediated inhibition of activator protein-1 transactivation. *Biochem. Pharmacol.* **69**, 1785-1794
25. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J. S. (1992) Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**, 1031-1040
26. Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* **68**, 1041-1050
27. de Vries-Smits, A. M., Burgering, B. M., Leever, S. J., Marshall, C. J. and Bos, J. L. (1992) Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature* **357**, 602-604
28. Rubio, I., Rennert, K., Wittig, U. and Wetzker, R. (2003) Ras activation in response to lysophosphatidic acid requires a permissive input from the epidermal growth factor receptor. *Biochem. J.* **376**, 571-576
29. Meng, T. C., Fukada, T. and Tonks, N. K. (2002) Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol. Cell* **9**, 387-399
30. Bivona, T. G., Perez De Castro, I., Aheam, I. M., Grana, T. M., Chiu, V., Lockyer, P. J., Cullen, P. J., Pellicer, A., Cox, A. D. and Philips, M. R. (2003) Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. *Nature* **424**, 694-698
31. Buday, L. and Downward, J. (1993) Epidermal growth factor regulates the exchange rate of guanine nucleotides on p21ras in fibroblasts. *Mol. Cell. Biol.* **13**, 1903-1910
32. Ehrhardt, A., David, M. D., Ehrhardt, G. R. A. and Schrader, J. W. (2004) Distinct mechanisms determine the patterns of differential activation of H-Ras, N-Ras, K-Ras 4B, and M-Ras by receptors for growth factors or antigen. *Mol. Cell. Biol.* **24**, 6311-6323
33. Mifune, M., Ohtsu, H., Suzuki, H., Nakashima, H., Brailoiu, E., Dun, N. J., Frank, G. D., Inagami, T., Higashiyama, S., Thomas, W. G., Eckhart, A. D., Dempsey, P. J. and Eguchi, S. (2005) G protein coupling and second messenger generation are indispensable for metalloprotease-dependent, heparin-binding epidermal growth factor shedding through angiotensin II type-1 receptor. *J. Biol. Chem.* **280**, 26592-26599
34. Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995) Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6**, 967-980

35. Datta, R., Yoshinaga, K., Kaneki, M., Pandey, P. and Kufe, D. (2000) Phorbol ester-induced generation of reactive oxygen species is protein kinase c $\beta$  -dependent and required for SAPK activation. *J. Biol. Chem.* **275**, 41000-41003
36. Zor, U., Ferber, E., Gergely, P., Szucs, K., Dombradi, V. and Goldman, R. (1993) Reactive oxygen species mediate phorbol ester-regulated tyrosine phosphorylation and phospholipase A2 activation: potentiation by vanadate. *Biochem. J.* **295**, 879-888
37. Arozarena, I., Matallanas, D., Berciano, M. T., Sanz-Moreno, V., Calvo, F., Munoz, M. T., Egea, G., Lafarga, M. and Crespo, P. (2004) Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors. *Mol. Cell. Biol.* **24**, 1516-1530
38. Porfiri, E. and McCormick, F. (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. *J. Biol. Chem.* **271**, 5871-5877
39. Langlois, W. J., Sasaoka, T., Saltiel, A. R. and Olefsky, J. M. (1995) Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated p21ras activation. *J. Biol. Chem.* **270**, 25320-25323
40. Holt, K. H., Kasson, B. G. and Pessin, J. E. (1996) Insulin stimulation of a MEK-dependent but ERK-independent SOS protein kinase. *Mol. Cell. Biol.* **16**, 577-583
41. Douville, E. and Downward, J. (1997) EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2. *Oncogene* **15**, 373-383
42. Duan, R.-D., Zheng, C.-F., Guan, K.-L. and Williams, J. A. (1995) Activation of MAP kinase kinase (MEK) and Ras by cholecystokinin in rat pancreatic acini. *Am. J. Physiol.* **268**, G1060-1065
43. Rubio, I. and Wetzker, R. (2000) A permissive function of phosphoinositide 3-kinase in ras activation mediated by inhibition of GTPase-activating proteins. *Curr. Biol.* **10**, 1225-1228
44. Lorenzo, P. S., Kung, J. W., Bottorf, D. A., Garfield, S. A., Stone, J. C. and Blumberg, P. M. (2001) Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* **61**, 943-949
45. Quatela, S. E. and Philips, M. R. (2006) Ras signaling on the Golgi. *Curr. Opin. Cell Biol.* **18**, 162-167
46. Downward, J. (2003) Role of receptor tyrosine kinases in G-protein-coupled receptor regulation of Ras: transactivation or parallel pathways? *Biochem. J.* **376**, e9-10
47. Raab, G. and Klagsbrun, M. (1997) Heparin-binding EGF-like growth factor. *Biochim. Biophys. Acta* **1333**, F179-F199
48. Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F. and Inagami, T. (1998) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced

- mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.* **273**, 8890-8896
49. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K. and Matsuda, M. (2000) CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J. Biol. Chem.* **275**, 25488-25493
  50. Roberts, D. M., Anderson, A. L., Hidaka, M., Swetenburg, R. L., Patterson, C., Stanford, W. L. and Bautch, V. L. (2004) A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters. **24**, 10515-10528
  51. Ozaki, N., Miura, Y., Yamada, T., Kato, Y. and Oiso, Y. (2005) RasGRP3 mediates phorbol ester-induced, protein kinase C-independent exocytosis. *Biochem. Biophys. Res. Commun.* **329**, 765-771
  52. Rambaratsingh, R. A., Stone, J. C., Blumberg, P. M. and Lorenzo, P. S. (2003) RasGRP1 represents a novel non-protein kinase C phorbol ester signaling pathway in mouse epidermal keratinocytes. *J. Biol. Chem.* **278**, 52792-52801
  53. Caloca, M. J., Zugaza, J. L. and Bustelo, X. R. (2003) Exchange factors of the RasGRP family mediate Ras activation in the Golgi. *J. Biol. Chem.* **278**, 33465-33473
  54. Yang, Y., Li, L., Wong, G. W., Krilis, S. A., Madhusudhan, M. S., Sali, A. and Stevens, R. L. (2002) RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J. Biol. Chem.* **277**, 25756-25774
  55. Corbalan-Garcia, S., Yang, S. S., Degenhardt, K. R. and Bar-Sagi, D. (1996) Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2. *Mol. Cell. Biol.* **16**, 5674-5682
  56. Zhao, D., Letterman, J. and Schreiber, B. M. (2001) beta-Migrating very low density lipoprotein (beta VLDL) activates smooth muscle cell mitogen-activated protein (MAP) kinase via G protein-coupled receptor-mediated transactivation of the epidermal growth factor (EGF) receptor: effect of MAP kinase activation on beta VLDL plus EGF-induced cell proliferation. *J. Biol. Chem.* **276**, 30579-30588
  57. Sakai, T., de la Pena, J. M. and Mosher, D. F. (1999) Synergism among lysophosphatidic acid, beta1A integrins, and epidermal growth factor or platelet-derived growth factor in mediation of cell migration. *J. Biol. Chem.* **274**, 15480-15486
  58. Li, J., Kim, Y. N. and Bertics, P. J. (2000) Platelet-derived growth factor-stimulated migration of murine fibroblasts is associated with epidermal growth factor receptor expression and tyrosine phosphorylation. *J. Biol. Chem.* **275**, 2951-2958

59. Cunnick, J. M., Dorsey, J. F., Standley, T., Turkson, J., Kraker, A. J., Fry, D. W., Jove, R. and Wu, J. (1998) Role of tyrosine kinase activity of epidermal growth factor receptor in the lysophosphatidic acid-stimulated mitogen-activated protein kinase pathway. *J. Biol. Chem.* **273**, 14468-14475

## Figure Legends

### Figure 1 TPA activates Ras in COS-7 cells.

(A) Sub-confluent serum-starved COS-7 cells were challenged with 100 nM TPA. After indicated time periods, cells were lysed in the presence of the Ras binding domain (RBD) of the Ras effector Raf-1 fused to GST and processed for detection of active GTP-loaded Ras as described in the experimental section. Aliquots of the cell extracts were analyzed in parallel to ascertain equal loading. (B) Same setup as in A, except COS-7 cells were stimulated with the indicated concentrations of TPA for 10 min.

### Figure 2 TPA stimulates nucleotide exchange on Ras.

(A) Permeabilization assay for assessment of nucleotide exchange on Ras. Serum-starved COS-7 cells were permeabilized with Streptolysin O in the presence of [ $\alpha$ - $^{32}$ P]GTP. Ras proteins were immunoprecipitated at various time points with the Y13-259 antibody in the presence or absence of 5  $\mu$ g/ml recombinant K-Ras protein, as indicated. [ $\alpha$ - $^{32}$ P]GTP incorporation was chased by addition of 100  $\mu$ M of indicated purine nucleotides at the time point marked by an asterisk. Washed immunoprecipitates were subjected to Cerenkov counting. (B) TPA elevates nucleotide exchange on Ras. Serum-starved COS-7 cells were challenged with 100 nM TPA 5 min or 1 min prior to permeabilization in the presence of [ $\alpha$ - $^{32}$ P]GTP and quenching of the first assay point (zero-time as recorded). Ras proteins were immunoprecipitated with Y13-259 at the indicated time points and radioactivity associated to immunoprecipitates was evaluated by Cerenkov counting. (C) Quantification of data presented in B. The amount of GDP + GTP bound to Ras 6 min after quenching the zero-time assay point (as recorded in B) was plotted as the fold-increase of radioactivity bound to Ras in TPA-stimulated cells versus unstimulated cells. Data represent mean  $\pm$  S.E.M. of at least three independent experiments. (D) Separation of guanine nucleotides associated to Ras. Guanine nucleotides were eluted from the same immunoprecipitates as in B and separated by thin layer chromatography (TLC). Values for % GTP/(GDP + GTP) are given below the figure. Three independent experiments yielded similar results.

**Figure 3** Role of EGFR and PKC in the activation of the Ras/Erk pathway by TPA. Sub-confluent, serum-starved COS-7 cells were treated with AG1478 (200 nM for 5 min) and/or BIM (500 nM for 10 min) or left untreated and challenged with 100 nM TPA. At the indicated time points cells were lysed in the presence of GST-RBD and phosphatase inhibitors. The activation/phosphorylation status of Ras, EGFR, Shc and Erk was sequentially determined in the same lysates as described in the experimental section. Antibodies used for immunoprecipitation (IP) or immunoblotting (IB) are indicated on the left

side of the panel. Four independent experiments yielded similar results. P-Erk: anti-phospho-Erk; pY: anti-phosphotyrosine.

**Figure 4** Basal EGFR activity maintains basal Ras-GTP levels.

(A) EGFR inhibition disrupts basal Shc/Grb2 complex formation. Serum-starved COS-7 cells were treated with 200 nM AG1478 for 5 min or left untreated as indicated. Cells were challenged with 100 nM TPA or vehicle DMSO and lysed at various time points. Cell extracts were split and subjected to EGFR or Shc immunoprecipitation. Tyrosine phosphorylation of both proteins or Shc/Grb2 association were assessed using phosphotyrosine (pY) or Grb2 directed antibodies, respectively. (B) Cells were challenged as in A and cell extracts were subjected to a Ras-GTP pulldown assay. Activation of Erk was assayed out of the same lysates as described in the experimental section. (C) TPA induces shedding of endogenous proHB-EGF. Serum-starved COS-7 cells were challenged for the indicated time points with 100 nM TPA or the vehicle DMSO. The medium was collected and incubated with heparin-agarose to collect secreted heparin-binding proteins. Proteins associated to the heparin beads were separated by SDS-PAGE and probed by immunoblotting with an antibody directed against the shed HB-EGF portion (sHB-EGF; top panel). In parallel, cells were lysed and cell lysates were analyzed by western blotting for the presence of membrane-bound proHB-EGF species by immunoblotting with a serum directed to the intracellular domain of HB-EGF (lower panel). (D) HB-EGF shedding in response to TPA is mediated by PKC. Serum-starved COS-7 cells were treated with BIM (10 min, 500 nM) or left untreated and challenged with TPA as indicated. Endogenous HB-EGF shedding was assessed as in C. (E) Interruption of HB-EGF-mediated EGFR transactivation does not compromise Ras activation by TPA. Serum-starved COS-7 cells were treated with the diphtheria toxin mutant CRM197 (5 µg/ml for 30 min) to block HB-EGF function and challenged with 100 nM TPA or DMSO vehicle. At the indicated time points cell extracts were prepared, split and subjected to a Ras-GTP pulldown assay or EGFR immunoprecipitation. Samples were processed as described in the experimental section. (F) COS-7 cells were treated with 500 nM BIM for 10 min or left untreated prior to stimulation with 100 nM TPA. Samples were processed as described above for assessment of Ras-GTP formation and Erk activation. (G) Summary of effects of EGFR or PKC inhibition on Ras-GTP levels. Absolute Ras-GTP levels in experiments shown in B, E and F were quantified by densitometric analysis and are shown plotted on the left panel. The value for the unstimulated and untreated cells was set as 1. \*P < 0.05 compared with untreated. In the right panel, values are corrected for the basal Ras-GTP levels and plotted as the ratio absolute/basal Ras-GTP levels. Data represent mean ± S.E.M. of three independent experiments. V: Vehicle.



**Figure 5** EGFR does not mediate GEF activation in response to TPA.

(A) Serum-starved COS-7 cells were pre-incubated with 200 nM AG1478 for 5 min or left untreated. Cells were challenged with 100 nM TPA or left unstimulated, followed by permeabilization in the presence of [ $\alpha$ - $^{32}$ P]GTP. Determination of guanine nucleotides associated to Ras was performed as described previously. Note that TPA stimulation occurred 5 min prior to zero-time point as recorded in the plot. (B) Guanine nucleotides associated to Ras immunoprecipitates in A were separated by TLC. (C) Experiment was performed as described in A, except cells were pre-treated with 500 nM BIM for 10 min. (D) Guanine nucleotides associated to Ras immunoprecipitates from experiment shown in C. (E) Quantification of nucleotide exchange for experiments shown in A and C. Left panel: TPA-induced exchange. The amount of GDP + GTP bound to Ras 6 min after quenching the zero-time assay point (as recorded in A and C) was plotted as the fold-increase of radioactivity associated to Ras in TPA-stimulated cells versus unstimulated cells for the various inhibitor treatments. Right panel: basal exchange rate. The amount of GDP + GTP bound to Ras in inhibitor-treated cells 6 min after quenching the zero-time assay point (as recorded in A and C) was plotted as % of radioactivity associated to Ras in untreated cells. Data represent mean and S.E. of at least three independent experiments.

**Figure 6** TPA transactivates the EGFR via ROS.

(A) TPA induces reversible PTP inhibition. Serum-starved COS-7 cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min or challenged for various time periods with 100 nM TPA. Cells were lysed in the absence or presence of iodoacetic acid, as indicated, to irreversibly alkylate the catalytic cysteine of non-oxidized PTPs. Extracts were separated by SDS-PAGE on a gel that had polymerized in the presence of a radiolabeled PTP substrate. Following in-gel renaturation of PTPs, renatured and non-alkylated (i.e. reversibly oxidized) PTPs hydrolyze the  $^{32}$ P-labelled substrate and give rise to negative (white) bands upon exposure to film. (B) ROS production mediates EGFR transactivation by TPA. Serum-starved COS-7 cells were pretreated 20 min with 20 mM NAC or left untreated. Cells were challenged with 100 nM TPA for various time points or with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. Cells were lysed and lysates were split for individual assessment of EGFR phosphorylation, Ras-GTP formation and Erk activation as described above. IAA: iodoacetic acid.

**Figure 7** EGFR mediates AKT activation in response to TPA. Serum-starved COS-7 cells were pre-treated with AG1478 (200 nM, 10 min) or vehicle DMSO and stimulated with 100 nM TPA. Cell extracts were separated by SDS-PAGE and Akt phosphorylation on Ser473 was assayed with a phosphorylation-site specific antibody (upper panel). Blot was stripped and reprobed for total load of Akt protein with pan-Akt serum (lower panel). V: vehicle.

**Figure 8** COS-7 cells do not express RasGRP1 and RasGRP3.

(A) Ethidium bromide stain of total RNA from COS-7, Ramos and Jurkat cells (all 6 µg) and green monkey brain cortex (2 µg) prior to processing for the Northern blot analysis shown in B. Molecular size marker is indicated in kilobases (Kb).

(B) Total RNA samples shown in A were transferred to a nylon membrane and subjected to Northern hybridisation with the probes indicated below the panels. Films were overexposed to avoid low transcript levels in COS-7 cells passing undetected. Molecular size marker is indicated in kilobases (Kb).

(C) Cell lysates of COS-7, Ramos and Jurkat cells (30 µg protein each) were analysed by Western blotting for the presence of RasGRP1 or RasGRP3. The membrane was subsequently stained with Coomassie to ascertain equal loading (right panel). IB: immunoblot.

**Figure 9** TPA induces PKC-dependent phosphorylation of Sos.

(A) Serum-starved COS-7 cells were stimulated for the indicated time periods with 100 nM TPA. Cell extracts were split and assayed in parallel for Ras-GTP formation and Sos mobility shift. (B) Serum-starved COS-7 cells were treated with 200 nM AG1478, 50 µM PD98059, 500 nM BIM or vehicle DMSO (control) (all 10 min) followed by administration of 100 nM TPA or 10 ng/ml EGF. Cell extracts were prepared at the indicated time points and Sos mobility shift was assayed by Western blotting.

**Figure 10** Schematic representation of TPA signalling to Ras and the EGFR in COS-7 cells. For reasons of clarity, two populations of EGFR are shown, one driving basal Ras-GTP formation, the second being targeted and transactivated in response to TPA. However, it is important to note that this study does not provide any evidence for the existence of different EGFR populations. The exact point of entry of ROS into the pathway is unknown.

# Figure 1

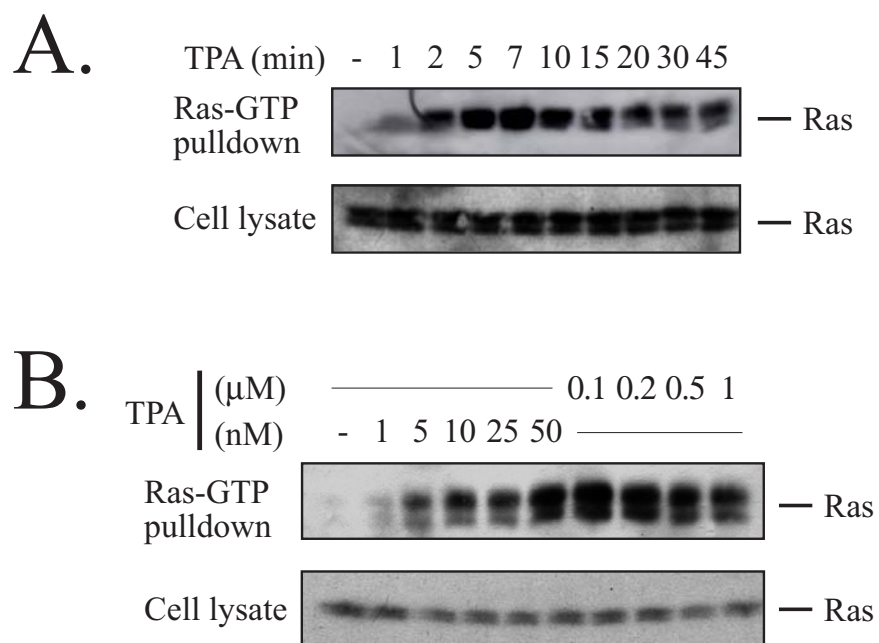


Figure 2

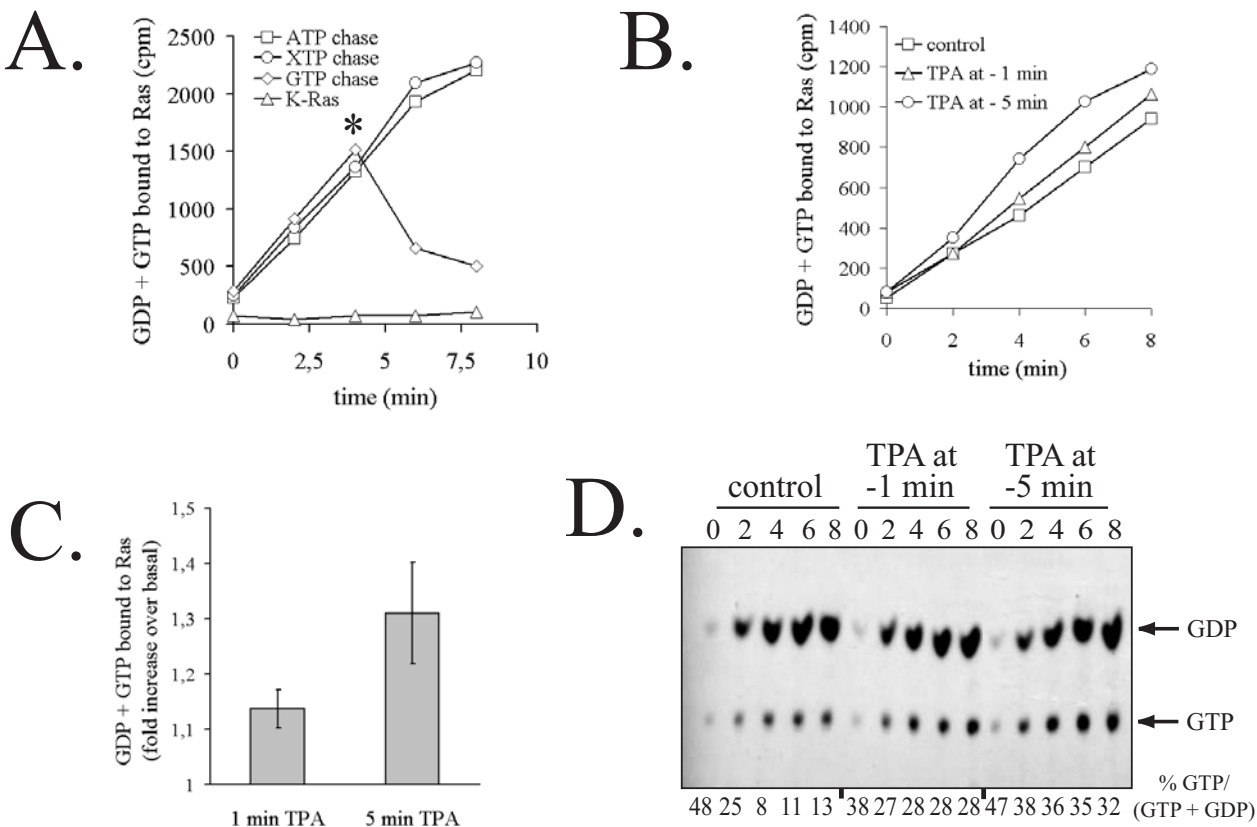
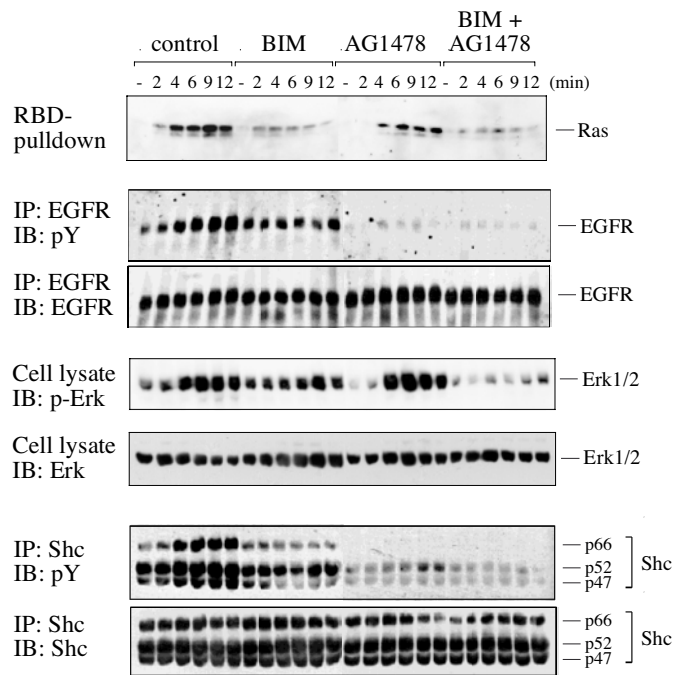
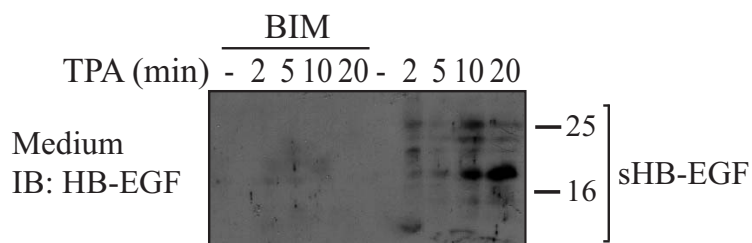


Figure 3

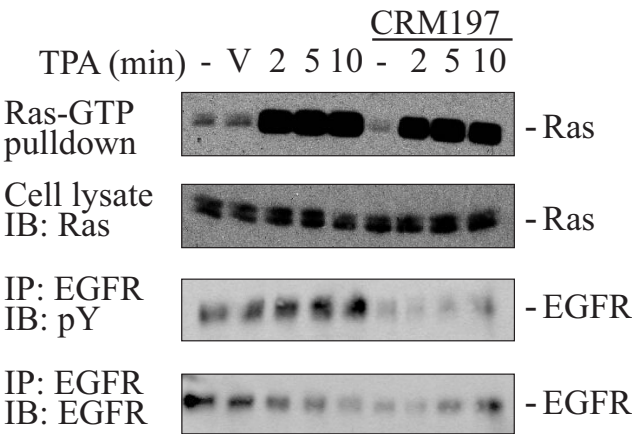


A.

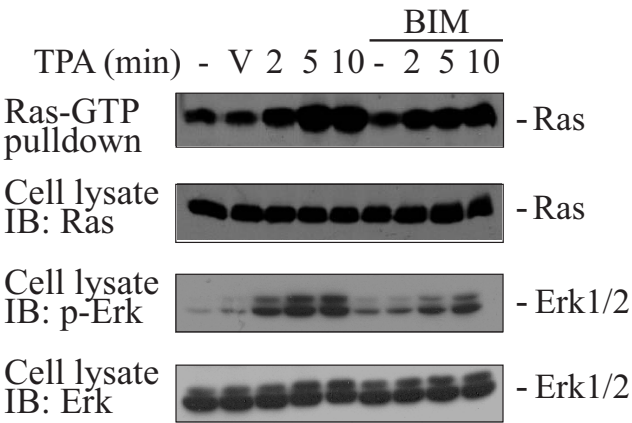


# Figure 4 continued

E.



F.



G.

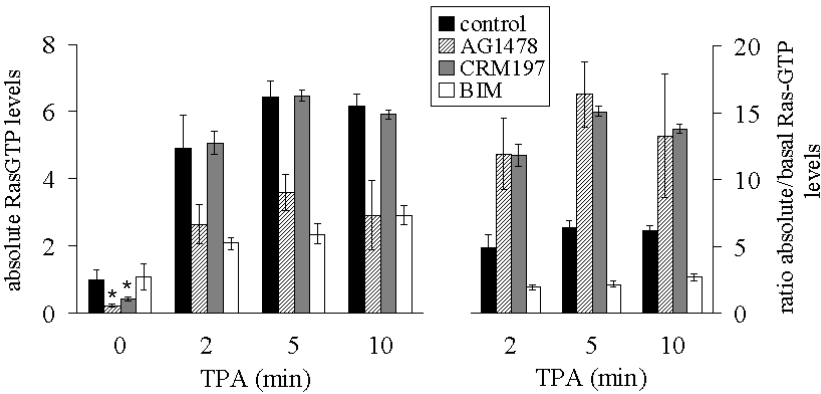
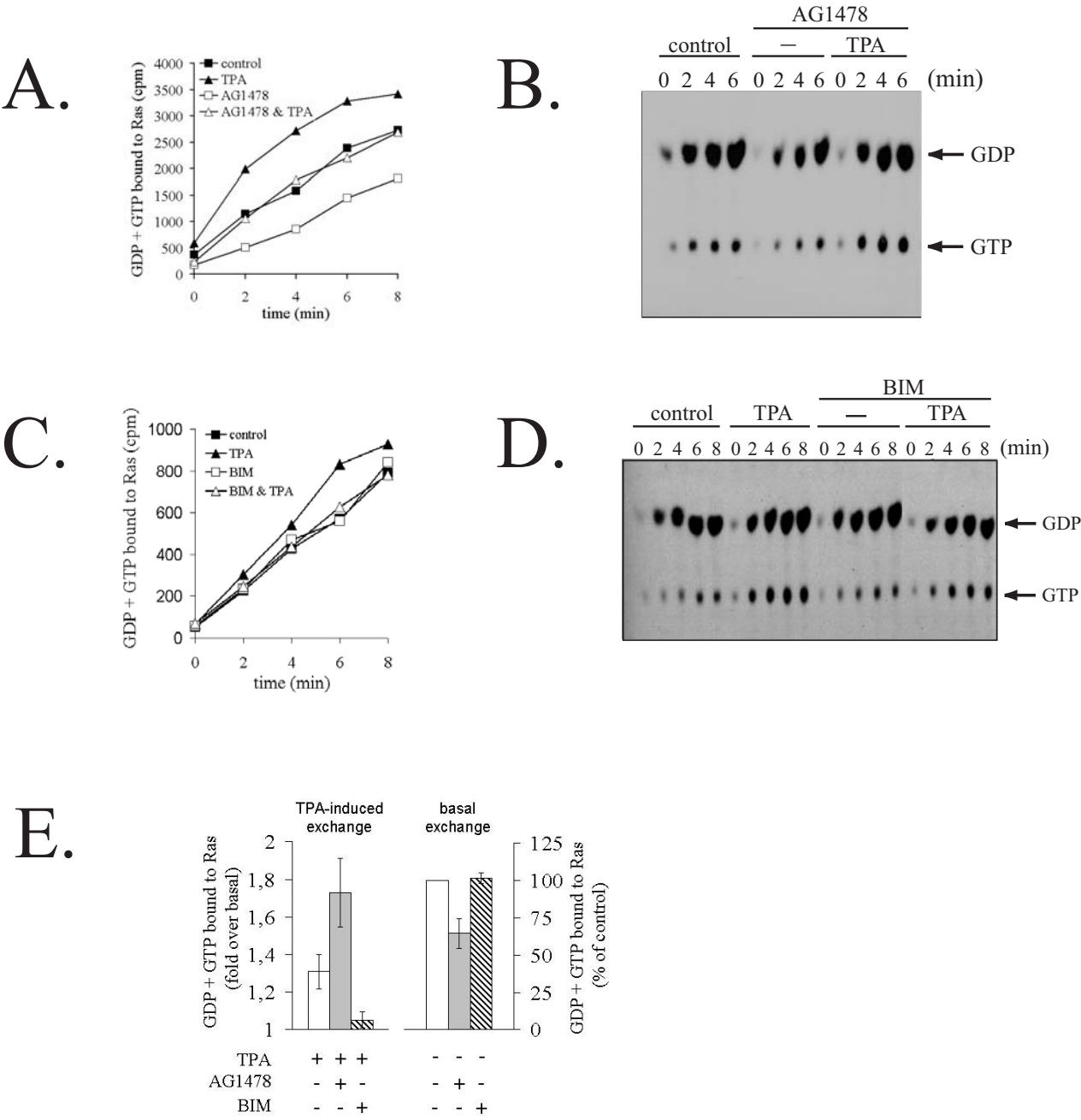
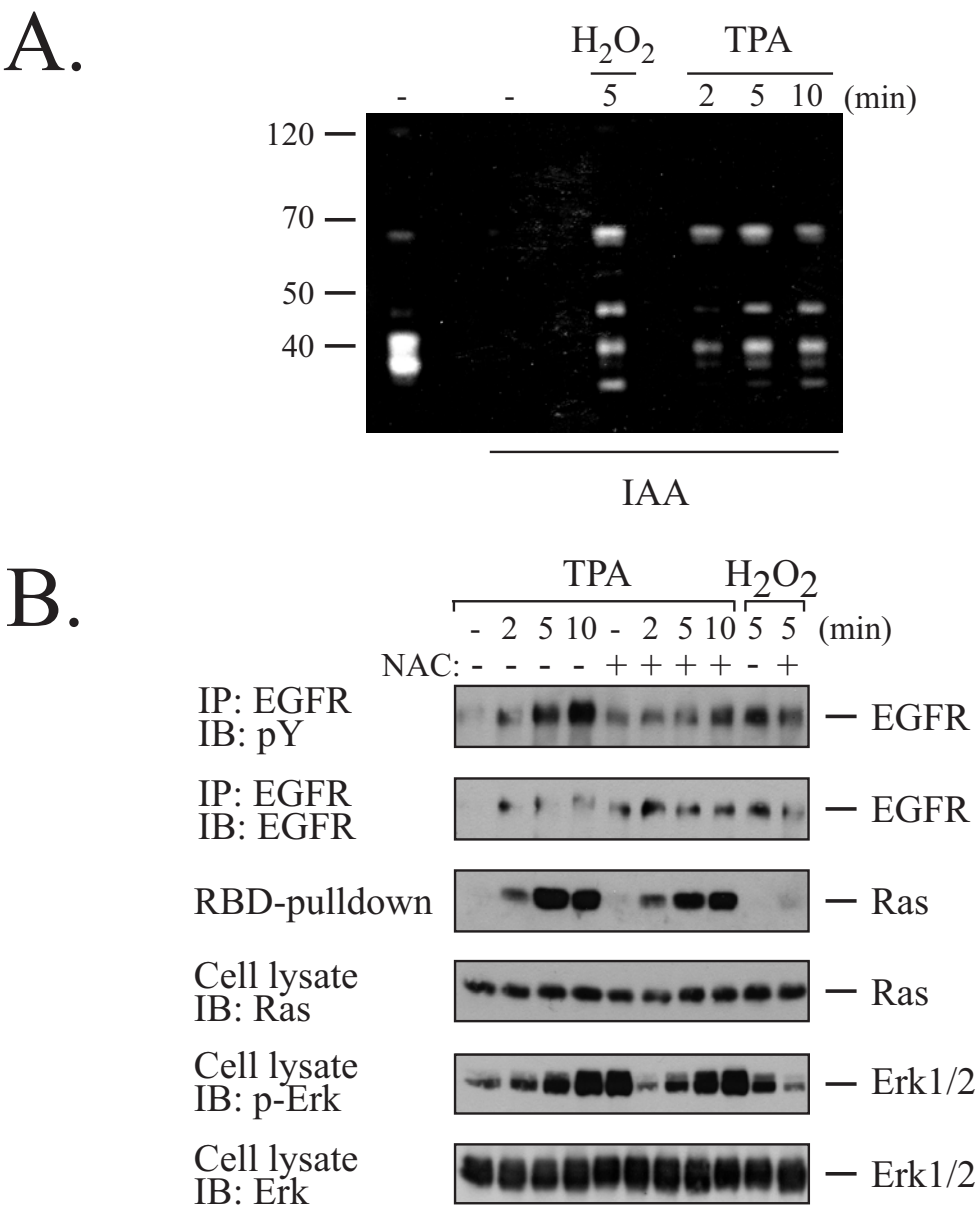


Figure 5

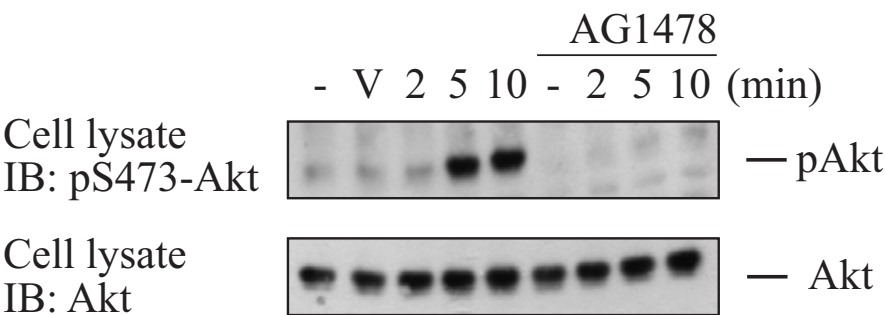




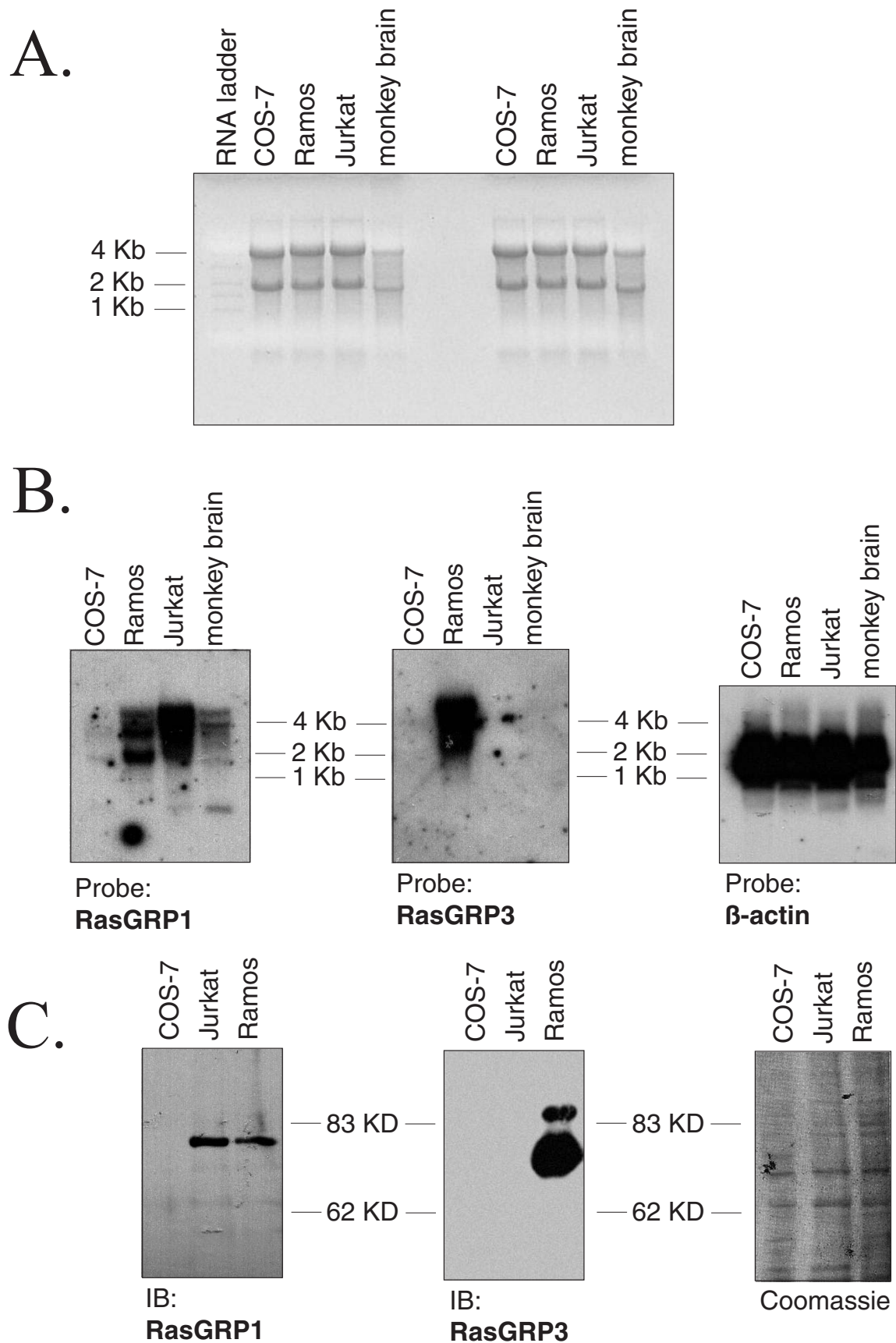
# Figure 6



# Figure 7



## Figure 8



# Figure 9

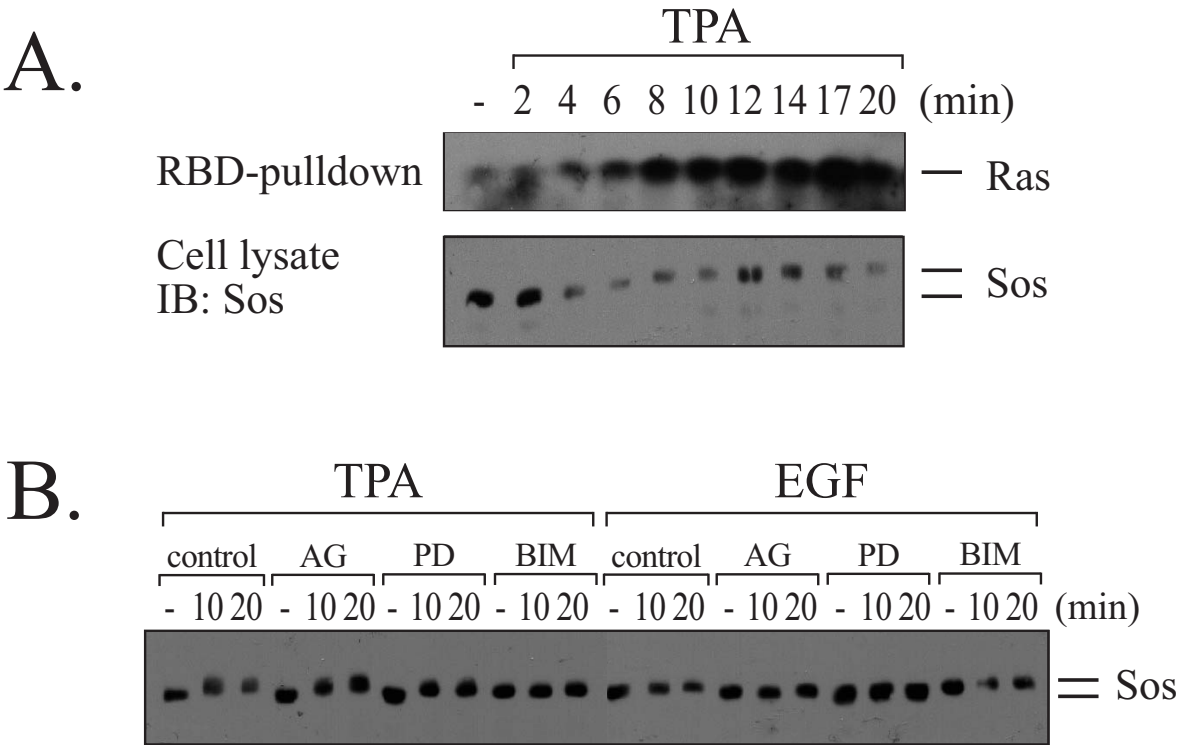


Figure 10

