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Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor–EGF receptor transactivation pathway

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In GN4 rat liver epithelial cells, angiotensin II (Ang II) produces intracellular calcium and protein kinase C (PKC) signals and stimulates ERK and JNK activity. JNK activation appears to be mediated by a calcium-dependent tyrosine kinase (CADTK). To define the ERK pathway, we established GN4 cells expressing an inhibitory Ras(N17). Induction of Ras(N17) blocked EGF- but not Ang II- or phorbol ester (TPA)-dependent ERK activation. In control cells, Ang II and TPA produced minimal increases in Ras–GTP level and Raf kinase activity. PKC depletion by chronic TPA exposure abolished TPA-dependent ERK activation but failed to diminish the effect of Ang II. In PKC-depleted cells, Ang II increased Ras–GTP level and activated Raf and ERK in a Ras-dependent manner. In PKC depleted cells, Ang II stimulated Shc and Cbl tyrosine phosphorylation, suggesting that without PKC, Ang II activates another tyrosine kinase. PKC-depletion did not alter Ang II-dependent tyrosine phosphorylation or activity of p125^{FAK}, CADTK, Fyn or Src, but PKC depletion or incubation with GF109203X resulted in Ang II-dependent EGF receptor tyrosine phosphorylation. In PKC-depleted cells, EGF receptor-specific tyrosine kinase inhibitors blocked Ang II-dependent EGF receptor and Cbl tyrosine phosphorylation, and ERK activation. In summary, Ang II can activate ERK via two pathways; the latent EGF receptor, Ras-dependent pathway is equipotent to the Ras-independent pathway, but is masked by PKC action. The prominence of this G-protein coupled receptor to EGF receptor pathway may vary between cell types depending upon modifiers such as PKC.

Keywords: EGF receptor/ERK/G-protein/PKC/tyrosine phosphorylation

Introduction

Multiple extracellular stimuli activate extracellular signal regulated protein kinases (ERK) including growth factors, phorbol esters and peptide hormones that activate G-protein coupled receptors (GPCRs). The mechanisms of activation by various stimuli may differ in part to control the strength and duration of the ERK response, which is involved in diverse cellular functions such as cell growth and differentiation. The growth factor receptor pathway is the best characterized (Marshall, 1995). For example, epidermal growth factor (EGF) triggers EGF receptor tyrosine autophosphorylation, resulting in a phosphotyrosine-dependent association of Shc and/or Grb2 with the receptor. Membrane translocation of the Grb2–SOS complex stimulates Ras-GDP to -GTP exchange, in turn stimulating Raf, MEK and ERK in series. More recently, alternative pathways involving other docking proteins and guanine nucleotide exchange factors have been studied. One major EGF receptor tyrosine phosphorylated, 120 kDa substrate, Cbl, has been implicated in Ras activation pathways in some cell types and can serve as a sensitive marker for EGF-dependent adaptor protein tyrosine phosphorylation (Fukazawa *et al.*, 1995; Galisteo *et al.*, 1995; Meisner and Czech, 1995; Tanaka *et al.*, 1995; Buday *et al.*, 1996; Panchamoorthy *et al.*, 1996; Reedquist *et al.*, 1996; Ribon *et al.*, 1996).

Another class of ERK activators, the protein kinase C (PKC) family of protein Ser/Thr kinases, are stimulated by the tumor promoter, TPA, or physiologically through activation of phospholipases C and D, which liberate diacylglycerol and inisotol triphosphate (IP3) or diacylglycerol alone, respectively. PKC stimulation activates ERK through several pathways depending on the cell type (Cobb and Goldsmith, 1995). The mechanisms, both Ras-dependent and independent, are not well understood. Specifically, the Ras-independent mechanisms by which PKC activates ERK, bypassing Raf kinase, have not been delineated (Post and Brown, 1996; van Biesen *et al.*, 1996).

Seven transmembrane GPCRs comprise the largest known family of cell surface receptors and they mediate cellular responses by a diverse array of ligands, including peptide and glycopeptide hormones, neurotransmitters, phospholipids, odorants, and even photons (van Biesen *et al.*, 1996). Activation of GPCRs catalyzes exchange of GTP for GDP within the heterotrimeric G-protein complex releasing activated Gα–GTP and Gβγ subunits, each of which has regulatory functions (Dhanasekaran *et al.*, 1995). As with PKC activation alone, the mechanism of GPCR ERK activation varies and has not been fully elucidated. In studying GPCRs in rat liver epithelial cells, we demonstrated angiotensin II (Ang II)-, vasopressin- and α_1 agonist-stimulated tyrosine phosphorylation (Huckle *et al.*, 1990; Huckle and Earp, 1992). We purified and identified the calcium-dependent tyrosine kinase (CADTK) (Earp *et al.*, 1995; Yu *et al.*, 1996) which was identified concurrently by others using different molecular approaches and has been named Pyk2 (Lev *et al.*, 1995), RAFTK (Avraham *et al.*, 1995), CAKβ (Sasaki *et al.*, 1995) and FAK2 (Herzog *et al.*, 1996). CADTK activation appears to regulate the c-Jun N-terminal kinase pathway (Zohn *et al.*, 1995; Tokiwa *et al.*, 1996; Yu *et al.*, 1996; Li *et al.*, 1997; Liu *et al.*, 1997) as well as cytoskeleton protein tyrosine phosphorylation, e.g. paxillin and p130^{CAS}

(Astier *et al.*, 1997; Li and Earp, 1997; Raja *et al.*, 1997). However, in GN4 cells, CADTK does not activate the ERK pathway, although it does in PC12 cells (Lev *et al.*, 1995).

The present study demonstrates that Ang II activates ERK in GN4 cells through two pathways. In control cells, Ang II stimulated ERK through what appears to be a PKC-dependent, Ras/Raf-independent pathway. However, when the PKC-dependent pathway was abolished, Ang II stimulated a new set of tyrosine phosphoproteins (e.g. Cbl) and still activated ERK to almost the same extent. Thus, PKC depletion uncovered an alternative Ras/Raf-dependent mechanism virtually as efficacious as the original. Several recent reports demonstrated cell-type specific, GPCR-dependent EGF receptor activation, e.g. endothelin stimulates EGF receptor and HER2/neu tyrosine phosphorylation in Rat-1 fibroblast cells (Daub *et al.*, 1996) and the m1 muscarinic acetylcholine receptor (mAChR) transactivates the EGF receptor in the m1 mAChR transfected human 293 cells (Tsai *et al.*, 1997). In this report, we demonstrate that Ang II and vasopressin stimulate EGF receptor tyrosine phosphorylation, but only when PKC is inhibited or depleted. PKC activation has long been known to decrease the affinity of the receptor for EGF and to block EGF-dependent signal transduction. The physiological reason for this PKC-dependent negative regulation has never been apparent, nor is the mechanism firmly established (Morrison *et al.*, 1996). Our current data suggest another important function for this negative regulation—prevention of a proliferative EGF receptor signal by ubiquitously expressed GPCRs. Agonists for these receptors are used for so many aspects of cell signaling that a linked proliferative signal with each engagement would be problematic. An intriguing question remains: 'When is the GPCR–EGF receptor interaction physiologically relevant?'

Results

Expression of dominant interfering Ras(N17) inhibits EGF- but not TPA-dependent ERK activation

Previously, we showed that Ang II and thapsigargin stimulated JNK in a calcium-dependent, PKC-independent manner (Zohn *et al.*, 1995). JNK activation was correlated with CADTK stimulation (Yu *et al.*, 1996). In contrast, in GN4 cells the calcium/CADTK-dependent pathway did not result in ERK activation (Yu *et al.*, 1996), nor did it increase c-Fos expression (Li *et al.*, 1997). Thus, we assumed that Ang II stimulated both ERK and AP-1 nuclear activity via a PKC-dependent pathway, and were surprised when Ang II retained the ability to increase ERK and AP-1 activity in cells depleted of PKC (Zohn *et al.*, 1995). To examine the role of Ras in this Ang II action, we established two clonal GN4 cell lines (S-5 and S-19), which expressed a dominant interfering form of the Ha-Ras protein, Ras(N17), under the control of an inducible promoter, the mouse mammary tumor virus (MMTV) long terminal repeat (Cai and Cooper, 1995). Treatment of stably transfected cells with dexamethasone (DEX) significantly increased Ras(N17) expression in these cells, whereas treatment with vehicle dimethysulfoxide (DMSO) did not (data not shown).

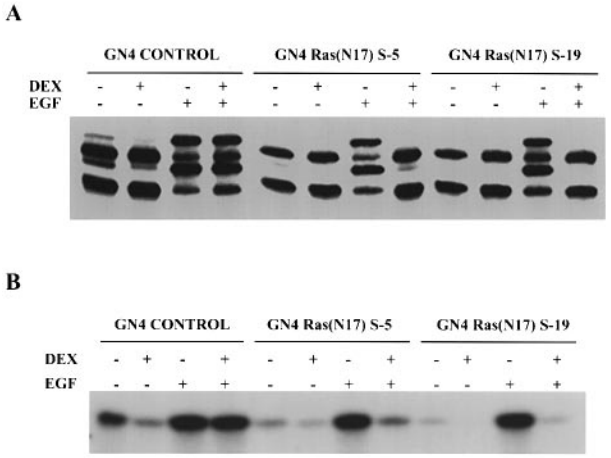

Fig. 1. EGF-dependent ERK activation is Ras-dependent. Rat liver epithelial cells (GN4) and Ras(N17) transfected GN4 cells (S-5, S-19) were pretreated with or without DEX (1 μM, 24 h) followed by stimulation with EGF (100 ng/ml, 5 min). Cell lysates were prepared and subjected to 15% SDS–PAGE followed either by (**A**) anti-ERK immunoblotting for ERK gel mobility shift or (**B**) by immunoprecipitating with anti-ERK antibody (C-l6) for ERK immune complex kinase activity assays with MBP as a substrate followed by gel electrophoresis and autoradiography to detect ^{32}P-MBP.

The consequence of Ras(N17) expression was tested by examining EGF-dependent ERK activation. As expected, EGF (100 ng/ml, 5 min) strongly activated ERK in control GN4 cells transfected with the vector alone, in either the presence or absence of DEX pretreatment. However, induction of Ras(N17) blocked EGF-dependent ERK activation (Figure 1A and B), confirming that induction of Ras(N17) in the S-5 and S-19 cell lines was sufficient to interfere with endogenous Ras signaling.

To define the TPA-dependent pathway in GN4 cells, ERK activation was again studied in the Ras(N17)-inducible cell lines. TPA activated ERK similarly in GN4 cells, with or without induction of Ras(N17) (Figure 2A and B). However, when PKC was depleted by chronic exposure of GN4 cells to TPA (5 μM, 18 h), the TPA-dependent activation of ERK was nearly abolished (Figure 2A and B). This result suggests that PKC activates ERK through primarily a Ras-independent pathway in GN4 cells. While expression of Ras(N17) does not block all potential mechanisms by which Ras can be activated (i.e. suppression of GAP activity), this type of experiment generally delineates Ras-dependent and -independent pathways. To investigate this further, we studied Ras-GDP to -GTP exchange and Raf activation.

Ang II activates ERK through two independent pathways

Like TPA, Ang II activated ERK in control cells whether or not Ras(N17) was induced (Figure 2C and D), suggesting that Ang II activates ERK through a Ras-independent, PKC-dependent pathway. However, in contrast with TPA, PKC-depletion did not appreciably inhibit the activation of ERK by Ang II (Figure 2C and D). Intriguingly, Ras(N17) induction in PKC-depleted cells inhibited Ang II-dependent ERK activation (Figure 2C and D). These data suggest that Ang II is capable of stimulating ERK by two pathways: in naive cells, Ang II activated ERK via a PKC-dependent, Ras-independent

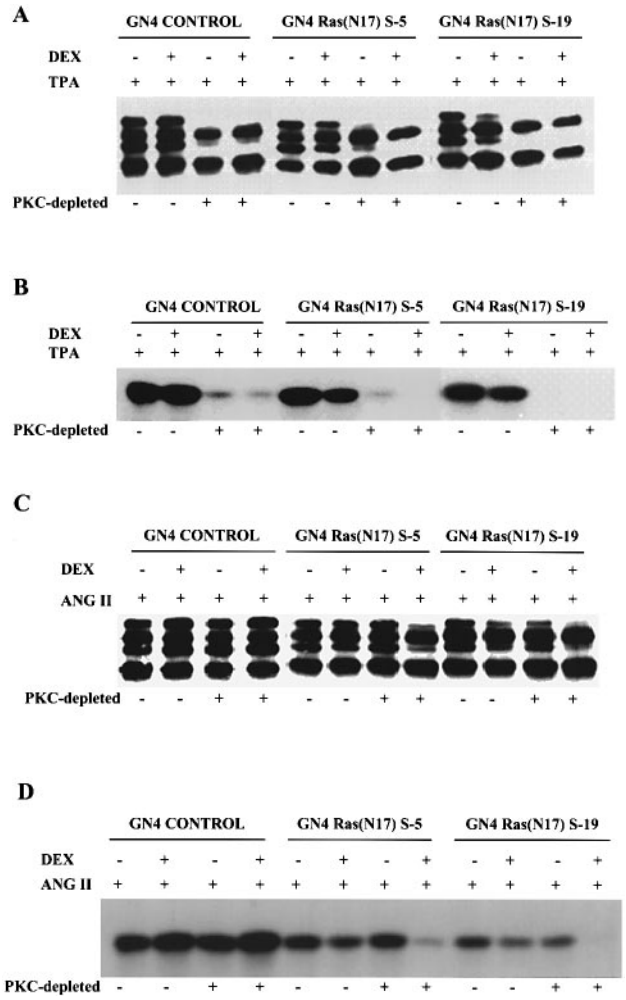

Fig. 2. Assessment of Ras and PKC-dependence of TPA and Ang II-dependent ERK activation. GN4 cells and Ras(N17) transfected GN4 cells (S-5, S-19) were pretreated with or without DEX (1 μM, 24 h) and TPA (5 μM, 18 h) or both, followed by acute treatment with TPA (100 nM, 5 min) or Ang II (1 μM, 5 min). Cell lysates were subjected to anti-ERK immunoblotting for gel mobility shift (**A** and **C**) or assessed for ERK activity by immune complex kinase activity assay (**B** and **D**) as described in Materials and methods. TPA-dependent ERK activation was not altered by DEX induction of Ras(N17) but was abolished by PKC depletion (A and B). Neither induction of Ras(N17) nor depletion of PKC alone blocked ERK activation by Ang II. However, the combination of both resulted in the inhibition of Ang II-dependent ERK activation, i.e. in PKC-depleted cells the pathway became Ras-dependent (C and D).

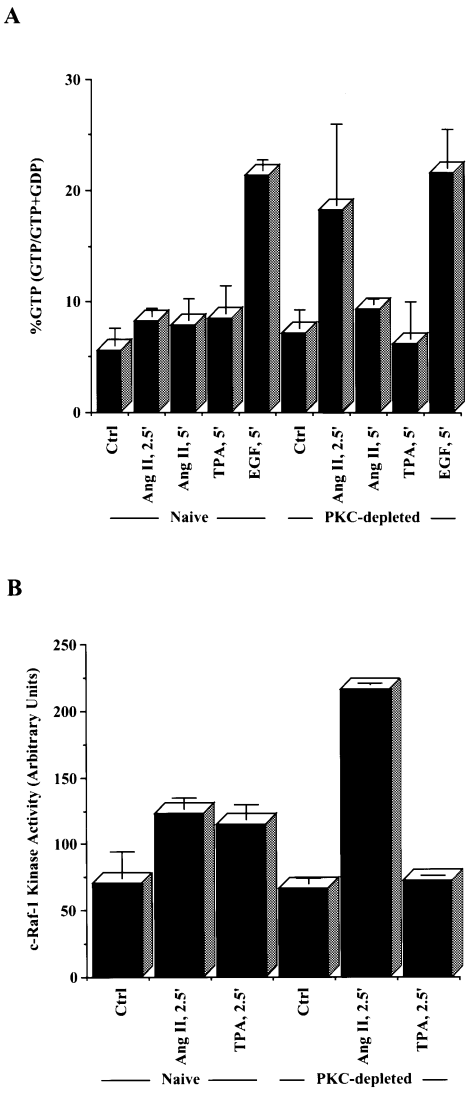

Fig. 3. Ang II increased Ras–GTP level and Raf kinase activity in PKC-depleted GN4 cells. GN4 cells pretreated with or without TPA (5 μM, 18 h) were stimulated by Ang II (1 μM), TPA (100 nM) and EGF (100 ng/ml) for the indicated time. Cell lysates were prepared and immunoprecipitated with (**A**) anti-Ha Ras or (**B**) anti-c-Raf antibodies. Ras–GTP level and c-Raf immune complex-kinase assays were then performed as described in Materials and methods. Ang II significantly activated Ras and Raf in PKC-depleted cells but TPA and Ang II-dependent effects in naive GN4 cells are minimal.

pathway; while in PKC-depleted cells, Ang II stimulated ERK via a Ras-dependent pathway.

The two pathways were further confirmed by measuring Ras–GTP level and Raf kinase activity in naive or PKC-depleted cells. Ang II and TPA treatment produced only minimal increases in Ras–GTP level in naive GN4 cells (Figure 3A). In contrast, Ang II significantly stimulated Ras–GTP level in PKC-depleted GN4 cells. While Ang II and TPA activated Raf kinase activity by a small increment (~60–70%) in naive GN4 cells, Ang II stimulated Raf kinase activity by an additional 2-fold in PKC-depleted GN4 cells (Figure 3B). The small effect of TPA was abolished by PKC depletion (Figure 3B) and the stimulation of Raf by Ang II in PKC-depleted cells was completely abrogated by induction of Ras(N17) (data not shown). The major increase in Ang II-dependent Ras–GTP level and Raf kinase activity in PKC-depleted cells confirms use of an alternative pathway. PKC-dependent ERK activation pathway predominates in naive rat liver epithelial cells while PKC depletion unmasks signaling through a Ras- and Raf-dependent pathway. Surprisingly, the two pathways yield nearly equivalent ERK (Figure 2) and MEK activation in these cells (data not shown). While we cannot rule out that a slight TPA-dependent increase in Ras–GTP level due to inhibition of GAP activity is involved in ERK activation, these data are most consistent with PKC acting via a Ras-independent pathway in these cell types.

Ang II treatment increases Shc and Cbl tyrosine phosphorylation

Shc is tyrosine phosphorylated in response to a number of stimuli and is thought to be a key intermediate in

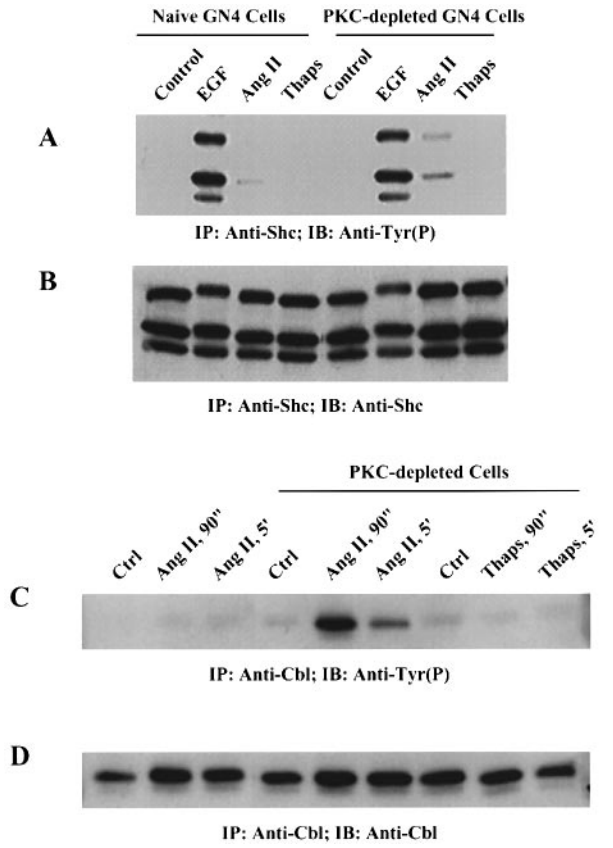

Fig. 4. Ang II increased Shc and Cbl tyrosine phosphorylation in PKC-depleted GN4 cells. GN4 cells with or without TPA pretreatment (5 μM, 18 h) were stimulated with EGF (100 ng/ml), Ang II (1 μM) or thapsigargin (2 μM) for 2.5 min. Cell lysates were prepared and immunoprecipitated with anti-Shc or anti-Cbl antibodies and the precipitates were subjected to SDS–PAGE. Immunoblot was probed with anti-Tyr(P) antibody (**A** and **C**), stripped, and reprobed with anti-Shc antibody (**B**) or anti-Cbl antibody (**D**).

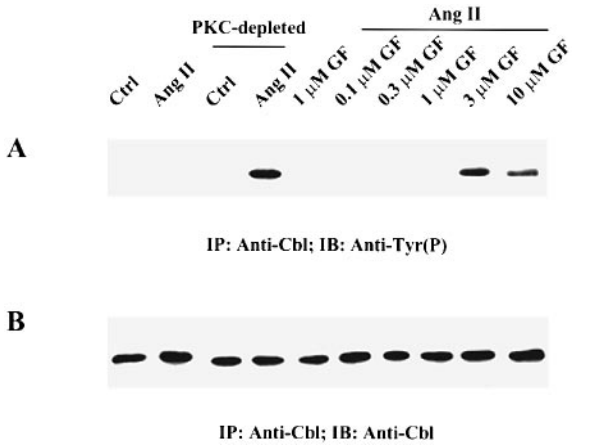

Fig. 5. Acute PKC inhibition with GF109203X mimics PKC depletion; both increased Ang II-dependent Cbl tyrosine phosphorylation. GN4 cells with or without various doses of GF109203X (15 min pretreatment) were stimulated with Ang II (1 μM, 90 s). Cell lysates were prepared and immunoprecipitated with anti-Cbl antibody. The immunoblot was probed with anti-Tyr(P) antibody (**A**), stripped, and reprobed with anti-Cbl antibody (**B**). At higher doses, GF109203X increased Ang II-stimulated Cbl tyrosine phosphorylation to an extent similar to that of PKC depletion caused by TPA (5 μM, 18 h) (lane 4).

growth factor receptor signaling to the Ras/Raf pathway (Pawson, 1995). As expected, EGF dramatically increased Shc tyrosine phosphorylation in GN4 cells, whereas Ang II did not (Figure 4A and B). However, in PKC-depleted cells Ang II slightly enhanced Shc tyrosine phosphorylation, although this effect was variable. In contrast, thapsigargin treatment, which stimulates CADTK, did not increase Shc tyrosine phosphorylation in either naive or PKC-depleted GN4 cells. Thus, CADTK is not the tyrosine kinase that phosphorylates Shc in Ang II-treated GN4 cells.

To investigate alternatives, we examined the tyrosine phosphorylation of Cbl, an adaptor protein which is a major EGF receptor substrate. The role of Cbl in Ras activation is somewhat controversial, but it has been implicated in some cells (Fukazawa *et al.*, 1995; Buday *et al.*, 1996; Panchamoorthy *et al.*, 1996; Reedquist *et al.*, 1996; Ribon *et al.*, 1996). Ang II treatment had little effect in naive GN4 cells, but consistently increased Cbl tyrosine phosphorylation in PKC-depleted cells (Figure 4C and D). Thapsigargin did not increase Cbl tyrosine phosphorylation in either naive or PKC-depleted GN4 cells. These data suggest that in PKC-depleted GN4 cells, an additional tyrosine kinase is now activated, stimulating Shc and Cbl tyrosine phosphorylation.

One obvious question is whether the effect of overnight TPA treatment is simply depletion (inactivation) of PKC, or whether prolonged TPA alters gene expression leading to the Ang II-dependent tyrosine phosphorylation pathway. To address this, we repeated the experiments on cells in which PKC activity has been acutely inhibited by treatment with GF109203X. Incubation with GF109203X produced a dose- and time-dependent (Figure 5; data not shown) increase in Ang II-dependent Cbl tyrosine phosphorylation, suggesting that it was a lack of PKC activation that allowed Ang II-dependent Cbl tyrosine phosphorylation.

Ang II-dependent tyrosine kinase activation

To determine whether PKC-depletion affected the ability of Ang II to stimulate specific tyrosine kinases, we immunoprecipitated tyrosine kinases from control and PKC-depleted GN4 cells and studied either their tyrosine autophosphorylation or their tyrosine kinase activity. The tyrosine autophosphorylation of $p125^{FAK}$ was unaffected by PKC depletion, while Ang II-dependent CADTK tyrosine phosphorylation was slightly decreased (Figure 6). Src or Src-family tyrosine kinases have been reported to stimulate Shc and/or Cbl tyrosine phosphorylation in response to GPCRs in some cell lines, and increased Src family kinase activity has often been correlated with ERK activation (Fukazawa *et al.*, 1995; Dikic *et al.*, 1996; Sadoshima and Izumo, 1996). However, immunoprecipitation of Src and Fyn from control or PKC-depleted cells followed by phosphotyrosine immunoblotting failed to detect any reproducible difference (Figure 6). Next, immune complex tyrosine kinase assays were performed using Src and Fyn immunoprecipitates from control and PKC-depleted cells, both with and without Ang II stimulation. Using poly($Glu^{80}Tyr^{20}$) as a substrate, Src and Fyn tyrosine kinase activity was slightly increased ($<$1.3-fold) in response to Ang II treatment; however, the increases in Src and Fyn kinase activity were similar in both naive and PKC-depleted GN4 cells (data not shown). Thus, Src and Fyn are probably not the key intermediates in Ang

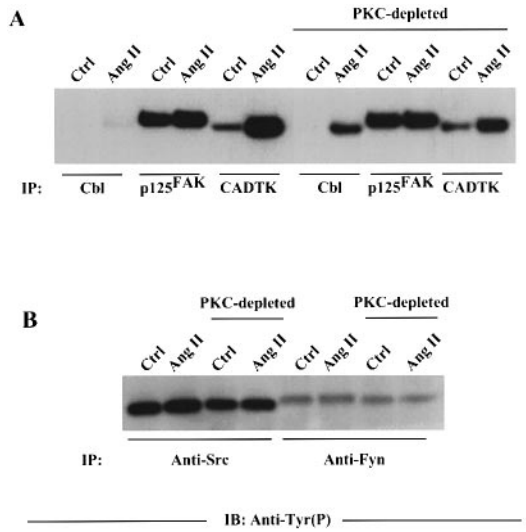

Fig. 6. PKC-depletion did not increase $p125^{FAK}$, CADTK, Src or Fyn tyrosine phosphorylation in response to Ang II treatment. GN4 cells with or without TPA pretreatment (5 μM, 18 h) were stimulated with Ang II (1 μM, 90 s). Cell lysates were prepared, immunoprecipitated with anti-Cbl, $p125^{FAK}$, CADTK, Src and Fyn, respectively, and immunoblotted with anti-Tyr(P) antibody. (**A**) and (**B**) show that PKC-depletion did not appreciably alter the extent of $p125^{FAK}$, CADTK, Src and Fyn tyrosine phosphorylation in response to Ang II.

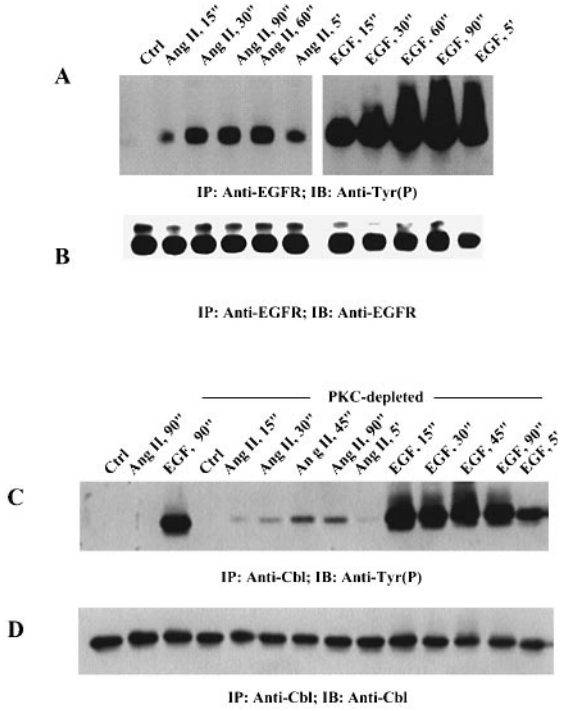

Fig. 7. The time course of Ang II- and EGF-stimulated EGF receptor and Cbl tyrosine phosphorylation in PKC-depleted GN4 cells. PKC-depleted (5 μM, 18 h, TPA) GN4 cells were treated with Ang II (1 μM) or EGF (100 ng/ml) for the indicated time. Cell lysates were prepared and immunoprecipitated with anti-EGF receptor (1382) (**A** and **B**) or anti-Cbl (**C** and **D**). The immunoblot was probed with anti-Tyr(P) (A and C), then stripped and reprobed with anti-EGF receptor (#22) (B) or anti-Cbl antibody (D). EGF dramatically increased Cbl tyrosine phosphorylation in as little as 15 s; Ang II-dependent Cbl tyrosine phosphorylation was delayed by 15–30 s with the peak at ~45–90 s. The exposure time for the EGF treated EGF receptor anti-Tyr(P) blot was shorter than that of the Ang II-dependent EGF receptor phosphorylation to allow better visualization of the time courses.

II-dependent Cbl tyrosine phosphorylation or ERK activation in PKC-depleted GN4 cells.

Ang II increased EGF receptor tyrosine phosphorylation resulting in ERK activation in PKC-depleted cells

Shc and Cbl are both EGF receptor substrates and therefore we examined Ang II-dependent EGF receptor tyrosine phosphorylation. Ang II treatment had little effect on EGF receptor tyrosine phosphorylation in naive GN4 cells but produced a time-dependent increase in EGF receptor tyrosine phosphorylation in PKC-depleted GN4 cells (Figure 7A and B). The same effect was observed when cells were treated with [Arg_8] vasopressin (data not shown), suggesting that multiple Gq protein-coupled receptors transactivate the EGF receptor in PKC-depleted GN4 cells. Both Ang II-dependent EGF receptor and Cbl tyrosine phosphorylation lagged 15–30 s behind that of EGF, as would be expected from a less direct activation mechanism (Figure 7A and C). The extent of EGF receptor tyrosine phosphorylation is substantially less with Ang II than with EGF, but it should be noted that GN4 cells express ~300 000 EGF receptors per cell (Tsao *et al.*, 1986); activation of 5–10% of this receptor number is probably sufficient to produce a biological effect (Figure 10).

To investigate further whether Ang II stimulation increased the EGF receptor tyrosine phosphorylation directly or whether a secondary kinase resulted in EGF receptor tyrosine phosphorylation, we incubated cells with several EGF receptor-specific inhibitors, such as PD153035 (Figure 8) and PD 158780 (data not shown) (Fry *et al.*, 1994). These compounds completely blocked EGF- and Ang II-stimulated Cbl tyrosine phosphorylation in PKC-depleted GN4 cells (Figure 8A). Both inhibitors, PD153035 (Figure 8C) and PD 158780 (data not shown), blocked EGF and Ang II-dependent EGF receptor tyrosine phosphorylation in PKC-depleted GN4 cells. These experiments using selective EGF receptor tyrosine kinase inhibitors suggest that GPCRs stimulate Cbl tyrosine phosphorylation via an EGF receptor mediated mechanism in PKC-depleted rat liver epithelial cells. (Since Ang II-stimulated EGF receptor tyrosine phosphorylation took only 15–30 s, it is unlikely that release of an autocrine ligand is involved in this process.)

Furthermore, we examined whether an Ang II EGF receptor pathway might explain the original biological observation of Ang II- and Ras/Raf-dependent ERK activation in PKC-depleted cells. Ang II and EGF increased ERK gel mobility shift in naive and PKC-depleted GN4 cells (Figure 9). PD153035 pretreatment completely blocked both the Ang II- and EGF-dependent effects in PKC-depleted cells. As expected, the selective EGF receptor tyrosine kinase inhibitor blocked the effect of EGF in naive cells but did not block the Ang II-dependent ERK activation (Figure 9, right three lanes), confirming the EGF receptor-independent nature of Ang II action in naive cells.

Finally, having established that Ang II-dependent EGF receptor transactivation was necessary for ERK activation in PKC depleted cells (Figure 9), we attempted to examine whether this transactivation was sufficient to activate ERK.

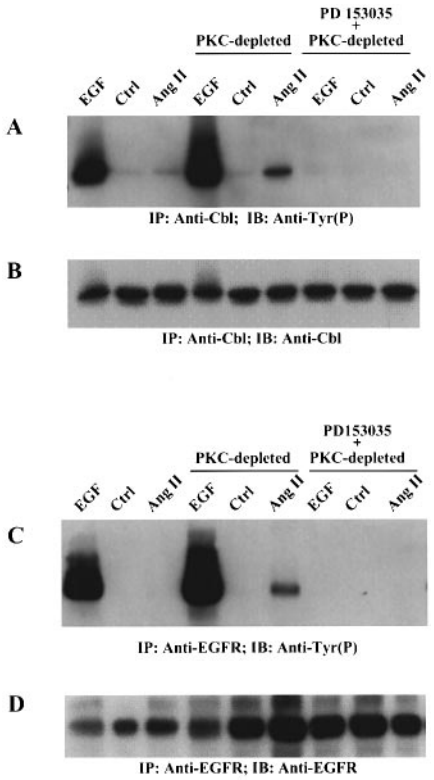

Fig. 8. PD153035 abolished Ang II-dependent EGF receptor and Cbl tyrosine phosphorylation in PKC-depleted cells. GN4 cells with or without TPA pretreatment (5 μM, 18 h) were treated with PD153035 (1 μM, 2 h) then stimulated with EGF (100 ng/ml, 90 s) or Ang II (1 μM, 90 s), respectively. Cell lysates were immunoprecipitated with anti-Cbl antibody or anti-EGF receptor (1382) antibody, then subjected to SDS–PAGE and immunoblotted with anti-Tyr(P) antibody. Pretreatment of PD153035 completely inhibited EGF and Ang II-mediated Cbl (**A** and **B**) and EGF receptor (**C** and **D**) tyrosine phosphorylation. The same results were obtained with PD 158780 (data not shown).

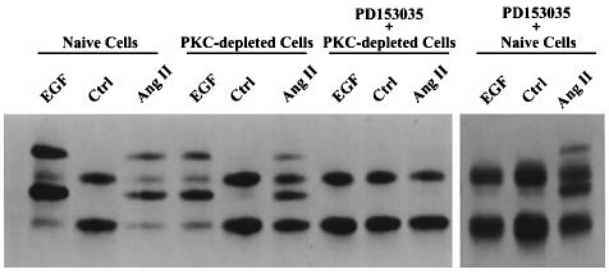

Fig. 9. PD153035 abolished Ang II-dependent ERK activation in PKC-depleted but not in control cells. GN4 cells with or without TPA (5 μM, 18 h) and PD153035 (1 μM, 2 h) pretreatment were stimulated with EGF (100 ng/ml, 90 s) or Ang II (1 μM, 90 s). Cell lysates were prepared and subjected to SDS–PAGE, then immunoblotted with anti-ERK antibody. EGF and Ang II increased ERK gel mobility shift in both naive and PKC-depleted GN4 cells. However, Ang II-stimulated ERK gel shift was only inhibited with PD153035 treatment in PKC-depleted cells; the Ang II effect in naive cells was not blocked by PD15035 (outside right lane). EGF-dependent ERK activation was abolished by PD153035 under all conditions.

Ang II (1 μM) treatment induced EGF receptor tyrosine phosphorylation that was similar to that produced by 1 ng/ml EGF (Figure 10A). Ang II (1 μM) and EGF (1 ng/ml) treatment induced a similar amount of Cbl tyrosine phosphorylation in these cells (Figure 10C). Ang II and 1–2 ng/ml EGF treatment also resulted in similar

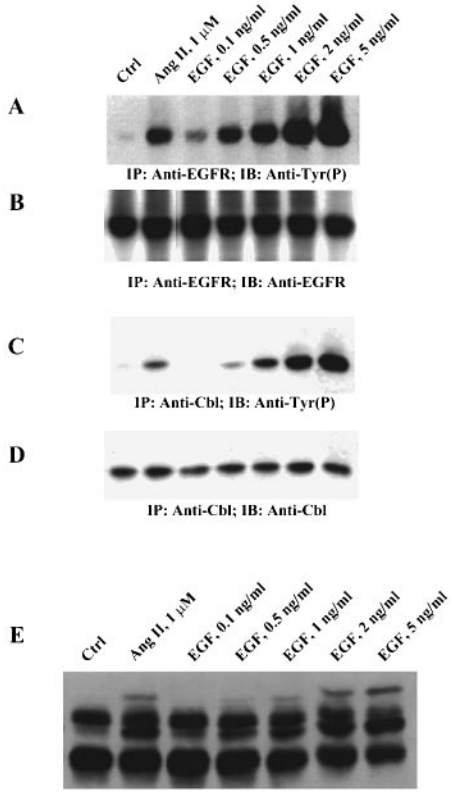

Fig. 10. Ang II-induced EGF receptor transactivation was sufficient to activate downstream signaling. PKC-depleted GN4 cells were treated with Ang II (1 μM) and EGF as indicated and concentrated for 90 s, respectively. Cell lysates were immunoprecipitated with anti-EGF receptor and anti-Cbl antibodies, respectively, and then subjected to SDS–PAGE and immunoblotted with anti-Tyr(P) antibody (A and C). Immunoblots were stripped and reprobed with (**B**) anti-rat EGF receptor or (**D**) anti-Cbl. Cell lysates were also subjected to SDS–PAGE and immunoblotted with anti-ERK antibody (**E**). One μM Ang II treatment stimulated EGF receptor (**A**) and Cbl (**C**) tyrosine phosphorylation to the same extent as 1 ng/ml EGF. ERK activation by Ang II was equivalent to 1–2 ng/ml EGF (E).

amounts of ERK activation (Figure 10E), suggesting that Ang II-induced EGF receptor transactivation was necessary and sufficient to activate downstream signaling.

Discussion

While it is widely accepted that GPCRs regulate ERKs (Post and Brown, 1996), the pathways used are not always defined and may vary between cell types or even receptor subtypes (Post and Brown, 1996; van Biesen *et al.*, 1996). Our studies demonstrate the following in rat liver epithelial cells: first, Ang II can stimulate ERK via two independent pathways, a putative Ras/Raf-independent, PKC-dependent pathway in naive GN4 cells and a Ras/Raf-dependent pathway observed when PKC activation is prevented. Secondly, the latter pathway may involve adaptor protein tyrosine phosphorylation as exemplified by Cbl and Shc (Figure 4). Tyrosine phosphorylation of these proteins is only seen in the absence of PKC activity. Thirdly, the Ang II, Ras-dependent pathway in PKC-depleted cells appears to involve the EGF receptor. Ang II stimulated EGF receptor and Cbl tyrosine phosphorylation in PKC-depleted GN4 cells (Figure 7); both these effects were abolished by selective EGF receptor tyrosine kinase inhib-

itors as was Ang II-dependent, Ras/Raf-dependent ERK activation (Figures 8 and 9). Finally, Ang II-dependent transactivation of the EGF receptor appeared to be necessary and sufficient to activate downstream signaling, such as ERK (Figure 10).

The mechanism controlling the switch between these two independent ERK activation pathways is not yet known, but inhibition of PKC is a key factor. It has long been known that PKC can phosphorylate the EGF receptor (Cochet *et al.*, 1984; Iwashita and Fox, 1984) on threonine residue 654 (Hunter *et al.*, 1984; Davis and Czech, 1985) and that acute TPA treatment inhibits EGF receptor signaling function by several mechanisms. EGF binding to surface EGF receptors is reduced either by decreasing receptor affinity for EGF (~10-fold) or by triggering ligand-independent internalization of the receptor (Beguinot *et al.*, 1985; McCune and Earp, 1989). Receptor internalization into intracellular vesicles capable of recycling occurs in selected epithelial cells but not in most fibroblasts. TPA also downregulates EGF receptor signaling by inhibiting EGF-dependent EGF receptor tyrosine kinase activity (Cochet *et al.*, 1984; Friedmann *et al.*, 1984; McCune and Earp, 1989). The mechanism by which TPA acts remains controversial. Initial studies suggested the PKC-dependent phosphorylation of Thr 654 in the juxtamembrane region was sufficient to explain downregulation. More recent data suggest that in some cells, mutated EGF receptor (Thr 654 to Ala 654) can also be inhibited by TPA treatment, indicating an additional mechanism in those cells (Morrison *et al.*, 1996). The mechanism proposed for the Thr 654-independent inhibition involves MEK/ERK activation by TPA. We have not yet determined whether Thr 654 phosphorylation is a key event preventing Ang II-dependent EGF receptor transactivation in GN4 cells; however, MEK/ERK activation cannot be the inhibitory mechanism because both are fully activated in PKC-depleted cells (Figure 2; data not shown). Interestingly, and in contrast with our findings in rat liver epithelial cells, m1 mAChR-induced EGF receptor transactivation is a PKC-dependent process in human 293 cells (Tsai *et al.*, 1997). These latter data suggest that the mechanism of GPCR-induced EGF receptor transactivation varies in different tissues and cells. One possible explanation that must be explored is the differential role of the multiple PKC isoforms.

Ang II-dependent adaptor protein tyrosine phosphorylation (e.g. Cbl and Shc) in PKC-depleted GN4 cells may link the Ang II EGF receptor signal to the ERK pathway. While less is known about Cbl, first identified as the cellular homolog of the murine retrovirus Cas-NS-1 transforming gene (v-*cbl*) (Langdon, 1995), this EGF receptor substrate has been linked to the Ras pathway in some cells (Fukazawa *et al.*, 1995; Buday *et al.*, 1996; Panchamoorthy *et al.*, 1996; Reedquist *et al.*, 1996; Ribon *et al.*, 1996). By sequence analysis, Cbl contains many protein–protein interaction domains including an SH3, a leucine zipper domain, a Cys_3–His–Cys_3 ring finger and a recently identified phosphotyrosine-binding domain that interacts with cellular tyrosine kinases and potentiates their signaling (Bonita *et al.*, 1997). Cbl is rapidly tyrosine-phosphorylated in response to EGF receptor activation as well as by non-receptor tyrosine kinases on T and B cells (Langdon, 1995); Cbl may stimulate Ras through Crk (Buday *et al.*, 1996; Reedquist *et al.*, 1996) or Grb2 adaptor proteins (Fukazawa *et al.*, 1995; Meisner *et al.*, 1995; Buday *et al.*, 1996; Panchamoorthy *et al.*, 1996). With regard to the role of Shc, Ang II activates Ras by increasing Shc tyrosine phosphorylation in cardiac myocytes (Sadoshima and Izumo, 1996) and thrombin stimulates Shc tyrosine phosphorylation via a Gq-mediated pathway in growth-responsive CCL39 fibroblasts (Pumiglia *et al.*, 1995). In PC12, bradykinin activates Pyk2/CADTK, stimulates Shc tyrosine phosphorylation and results in Pyk2–Grb2 association (Lev *et al.*, 1995). This is not the case in GN4 cells; neither thapsigargin nor calcium ionophore, which activate CADTK, increased Cbl or Shc tyrosine phosphorylation or ERK activity.

In some cells, Src and Src-family tyrosine kinases may be required for GPCR/ERK activation. Stimulation of ERK by Gq-coupled m1 mAChRs is blocked in avian B lymphoma cell lines deficient in the Src-family tyrosine kinase, Lyn (Wan *et al.*, 1996). The GPCR-Pyk2/CADTK pathway to ERK in PC12 cells mentioned above is thought to involve a Pyk2 and Src complex. Interestingly, Csk activation, which inhibits ERK activation in PC12 cells (Dikic *et al.*, 1996), is required for Gq protein-coupled receptor activation of ERK in DT40 cells (Wan *et al.*, 1997). How GPCR activates Src or Src family tyrosine kinases is still not clear; however, expression Csk or dominant-negative Src kinase mutants inhibit Gi protein-coupled receptor (and $G\beta1\gamma2$ subunits-mediated) phosphorylation of both EGF receptor and Shc (Luttrell *et al.*, 1997). In GN4 cells, however, the Ang II-dependent increase in Src and Fyn tyrosine kinase activity was minimal and was not changed with PKC-depletion, suggesting that Src and Fyn play only a minor role, if any, in Ang II-dependent ERK activation in rat liver epithelial cells.

Stimulation of EGF receptor tyrosine phosphorylation by agents other than EGF family member ligands has been reported by several groups. Calcium ionophore produces this effect in cells of neural origin (Rosen and Greenberg, 1996; Zwick *et al.*, 1997), but thapsigargin does not stimulate EGF receptor phosphorylation in GN4 cells and, thus, calcium is not the intermediary in GN4 cells. As mentioned above, a pathway of EGF receptor phosphorylation from Gi-linked receptors involving Src family kinases has been suggested by overexpression of Gi and $\beta\alpha$ in Cos-7 cells (Luttrell *et al.*, 1997). Perhaps closest to our observation is the report by Ullrich and colleagues that endothelin-1 activates the EGF receptor as well as rat $p185^{neu}$ in Rat-1 fibroblast cell lines (Daub *et al.*, 1996). However, this was observed in Rat-1 cells without any attempt to modify PKC activity. Thus, either endothelin does not activate the PKC inhibitory pathway, or PKC is not a negative regulator in all cell types. A PKC isoform present in rat liver epithelial, and not in Rat-1 fibroblast, cell lines may be responsible for the GPCR→EGF receptor pathway inhibition; alternatively, endothelin may use a different positive signal between its receptor and the EGF receptor. The presence of a GPCR pathway to EGF receptor and ERK activation has implications for the manner in which this ubiquitous class of receptors regulates to proliferation and differentiation systems. It is not surprising that a mechanism prevents multiple GPCRs from committing the cell to a major

energy-expending process such as cell growth. Thus, the masking of this growth stimulating pathway by PKC makes physiological sense. The intriguing question (in addition to the mechanism by which this occurs) is why a G protein/EGF receptor pathway exists and under what physiological conditions it is operable.

Materials and methods

Materials

Human recombinant EGF was purchased from Gibco-BRL. Ang II was purchased from Sigma and prepared in 50 mM acetic acid as stock solution. Phorbol 12-myristate 13-acetate (TPA), thapsigargin and DEX were purchased from Sigma, GF 109203X from Calbiochem, prepared as stock solutions in DMSO. PD153035 and PD158780 were kindly provided by Dr David W.Fry (Parke-Davis). Anti-CADTK polyclonal antibodies were as described previously (Yu *et al.*, 1996). Anti-p125^{FAK} monoclonal antibody was kindly provided by Dr Tom Parsons (University of Virginia). Anti-Src monoclonal antibody (327) was generously provided by Dr Joan Brugge (Harvard University). Anti-Fyn polyclonal antibody was kindly provided by Dr Andre Veillette (McGill University). Anti-Tyr(P) monoclonal antibody, PT66, was purchased from Sigma. Anti-ERKs polyclonal antibodies (C-16 and K-23), anti-HaRas (259) AC, anti-Raf-1 polyclonal antibody (C-12) and anti-Cbl polyclonal antibody (C-15) were purchased from Santa Cruz Biotechnology. Anti-Shc polyclonal and anti-Grb2 monoclonal antibodies were purchased from Transduction Laboratory.

Cell culture

Rat liver epithelial cells, GN4, were grown in Richter's improved minimal essential medium as described previously (Earp *et al.*, 1995). Ras(N17) stable integrated GN4 cell lines were established by transfecting GN4 with the pMMTV-Ras(N17) plasmid by lipofectAMINE according to the manufacturer's instructions (Gibco-BRL). Transfected GN4 cells were selected by incubating cells with G418 containing medium (400 μg/ml). Colonies were picked using cloning rings.

Cell lysate preparation

Cell lysate preparation was performed essentially as described previously (Earp *et al.*, 1995). Briefly, cells treated with agonists were scraped into ice-cold cell lysis buffer [150 mM NaCl, 20 mM Tris pH 7.5, 1% Triton X-100, 5 mM EDTA, 50 mM NaF and 10% (v/v) glycerol] with freshly added 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin and 100 kallikrein inhibitor units of aproptinin per ml. Cell lysates were clarified by centrifugation and their protein content was determined by Coomassie protein assay reagent (Pierce).

Immunoprecipitation and immunoblotting

In a typical experiment, ~750 μg of cell lysate was immunoprecipitated by incubation with the antibody for 2 h at 4°C. Twenty μl of protein A agarose beads were then added for an additional 1 h. Immune complexes were collected by centrifugation, washed three times with lysis buffer and resuspended in SDS–PAGE sample buffer. Samples were subjected to SDS–PAGE, transferred to Immobilon (Millipore) and incubated with the selected antibody. Immunoblots were developed with ECL according to the manufacturer's instructions (Amersham). Immunoblots were stripped in the buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at 50°C for 30 min and reprobed with another antibody.

ERKs activation assays

Activation of ERKs was determined as described previously (Yu *et al.*, 1996). Briefly, 15 μg of cell lysates were resolved on 15% low bis SDS–PAGE. Proteins were then transferred to nitrocellulose membranes for analysis by immunoblotting with the anti-ERKs antibody (K-23). The immunoblot was developed by ECL according to the manufacturer's procedure (Amersham). ERKs activity were determined by immunoprecipitated ERKs with anti-ERKs antibody (C-16) from cell lysate. Immune complexes were then washed three times with lysis buffer and once with kinase buffer. Immune complexes kinase assay was performed using [γ-^{32}P]ATP and myelin basic protein (MBP) as substrates. Reaction mixtures were then subjected to 12% SDS–PAGE, followed by Coomassie Blue staining and autoradiography.

Ras–GTP level assay

Ras activation was determined as described previously (Downward *et al.*, 1991) with some modification. Briefly, naive or PKC-depleted GN4 cells (100 mm dish) were incubated with 1 mCi ^{32}P-orthophosphate for 2 h before agonists were added. After washing, the cells were scraped into 1 ml of Ras lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 5 mM $MgCl_2$, 1 mg/ml BSA, 10 mM benzamidine HCl, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM PMSF, 10 μM leupeptin and 10 μM pepstatin A). Supernates of cell lysates were incubated with anti-Ha Ras (259) AC for 2 h at 4°C. The beads were collected by brief microfugation and washed five times with washing buffer (50 mM HEPES pH 7.4, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton and 0.005% SDS). The bound nucleotides were eluted by adding 30 μl of freshly made elution buffer (2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP) and incubating at 68°C for 20 min. The supernates were spotted on polyethyleneimine–cellulose TLC plates (Sigma). The plates were developed in 0.8 M HCl/1.2 M ammoniumformate and analyzed by phosphoimager.

Raf-1 activity assay

One mg of cell lysates were used to immunoprecipitate c-Raf by anti-c-Raf antibody (C-12) and protein A agarose beads. The immune complexes were washed twice with lysis buffer and twice with ice-cold phosphate-buffered saline (PBS). The Raf-1 immune complex kinase activity was measured through a coupled enzyme assay combining MEK, ERK2 and MBP. Briefly, immune complexes were incubated with 0.5 μg of recombinant MEK and 10 μl of a cold ATP mixture (30 mM β-glycerophosphate, 60 mM HEPES pH 7.3, 4 mM EGTA, 1.5 mM dithiothreitol, 0.45 mM Na_3VO_4, 30 mM $MgCl_2$, 0.3 mM ATP and 0.3 mg/ml BSA) for 10 min at 30°C. The 1.25 μg of ERK2 was added and incubated for an additional 10 min. Finally, 20 μl of a hot ATP mixture (2 μCi of [γ-^{32}P]ATP, 10 μg of MBP, 30 mM β-glycerophosphate, 60 mM HEPES pH 7.3, 4 mM EGTA, 1.5 mM dithiothreitol, 0.45 mM Na_3VO_4, 30 mM $MgCl_2$ and 6 μg of BSA) was added and incubated for 10 min at 30°C before stopping the reaction by the addition of 20 μl of EDTA. The reaction mixtures were then centrifuged at 12 000 *g* for 1 min. Forty μl of each supernatant was spotted onto P81 paper. The papers were then washed five times (5–10 min) with 75 mM phosphoric acid, soaked briefly with 100% ethanol and air dried prior to performing liquid scintillation counting.

Tyrosine kinase activity assay

The immune complex tyrosine kinase assay was performed as reported previously (Huckle and Earp, 1992). Briefly, immune complex suspensions were preincubated for 5 min at 4°C with 160 μg of the synthetic tyrosine kinase substrate poly(Glu^{80}Tyr^{20}) (Sigma) or the control substrate poly(Glu). Reactions (80 μl total reaction volume) were initiated by adding of 5 μCi [γ-^{32}P]ATP. After 15 min at 25°C, 50 μl of the reaction mixture was spotted on P81 Whatman paper. The papers were washed once with 10 mM Na-pyrophosphate in 10% trichloroacetic acid and twice with 5% trichloroacetic acid, air-dried and assayed by liquid scintillation counting.

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