FEBS Letters 517 (2002) 206–210 FEBS 26014

Role of calmodulin in the modulation of the MAPK signalling pathway and the transactivation of epidermal growth factor receptor mediated by PKC

Francesc Tebar, Anna Lladó, Carlos Enrich*

Departament de Biologia Cellular, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Facultat de Medicina, Universitat de Barcelona, Casanova 143, 08036 Barcelona, Spain

Received 12 March 2002; accepted 15 March 2002

First published online 2 April 2002

Edited by Jesus Avila

Abstract We have recently shown that calmodulin (CaM) regulates the trafficking of epidermal growth factor receptor (EGFR) as well as the mitogen-activated protein kinase (MAPK) signalling pathway. However, the overall regulation of the MAPK pathway is achieved through a complex interplay of other several upstream effectors including G-proteins, EGF, EGFR, protein kinase C (PKC), phosphatidylinositol-3-kinase and CaM. In order to understand the role of CaM in the PKC-mediated transactivation of EGFR we have analysed the effect of a CaM antagonist, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide, on the 12-*O*-tetradecanoylphorbol-13-acetate-mediated activation of EGFR and the subsequent MAPK activation. The results show that CaM interferes with MAPK activation and the transactivation of EGFR mediated by PKC.

Key words: Epidermal growth factor receptor; Calmodulin; Protein kinase C; Transactivation; Calmodulin antagonist; Mitogen-activated protein kinase; Shedding

1. Introduction

Calmodulin (CaM) is the most abundant calcium-binding protein in non-muscle cells that function as second messenger in a variety of cellular processes including the organisation of cytoskeleton, cell proliferation [1–3] and membrane trafficking [4,5]. The recognition by Ca^{2+}/CaM of a short polypeptide segment in target proteins (CaM-binding proteins, CaMBPs) induces conformational changes in both CaM and the target, enabling the target protein to become functionally active [6]. The epidermal growth factor receptor (EGFR) is a CaMBP [7].

Using a CaM antagonist, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13), we have recently demonstrated a specific stimulation of tyrosine-phosphorylation of the EGFR despite the decrease in mitogen-activated protein kinase (MAPK) activity caused by interference on Raf-1 activity [5,8]. In addition, the W-13-mediated EGFR-phosphorylation was shown to be blocked by a metalloproteinase inhibitor (batimastat, BB94), indicating the possible involvement of shedding in this process [5]. The involvement of CaM in the regulation the MAPK pathway has been described at various levels: (1) it binds to EGFR and negatively regulates its tyrosine kinase activity [9]; (2) it also binds to Ras (K-Ras), modulating its downstream signalling [10]; (3) CaM through the CaM kinase II phosphorylates EGFR and thus inhibits its tyrosine kinase activity [11]. Nevertheless, the overall regulation of the MAPK signalling pathway is much more complex with a range of intermediate molecules involved (e.g. G-proteins, protein kinase C (PKC), phosphatidylinositol-3-kinase, EGFR/EGF or by EGFR transactivation) [12].

The interplay of CaM and PKC in the transactivation of EGFR remains unclear; PKC binds and phosphorylates kinases, such as EGFR, providing a mechanism for cross-regulating the phosphorylation state and its catalytic activity [13]; binding of CaM inhibits the PKC-mediated phosphorylation of EGFR [14,15]. Apparently, the PKC-induced phosphorylation of the EGFR at threonine-654 (Thr654), is sufficient to direct incoming receptors to the recycling endosomes, whereas phosphorylation at tyrosine residues directs the receptor to the MVB/late endosomal compartment [14], in agreement with the close relationship between endocytosis, trafficking, sorting and signalling events. Furthermore, CaM kinase II or extracellular factors (released by shedding) might regulate the transactivation of EGFR by PKC activation or by changes in the content (or re-distribution) of CaM [16,17]. The augmented transactivation of EGFR, through PKC, increases MAPK activation [16].

In the present study we show that in COS-1 cells CaM interferes with the transactivation of EGFR mediated by PKC and, consequently, inhibition of PKC partially blocks the CaM-dependent EGFR transactivation. CaM is also involved in the PKC-mediated activation of Ras.

2. Materials and methods

2.1. Reagents and antibodies

W-13, W-12 and tyrphostin (AG1478) were from Sigma (Madrid, Spain). A monoclonal antibody to the extracellular domain of the EGFR (Ab225) was obtained from the American Type Culture Collection (Rockville, MD, USA); the rabbit polyclonal antibodies

*Corresponding author. Fax: (34)-93-4021907.
E-mail address: enrich@medicina.ub.es (C. Enrich).

Abbreviations: EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGF-like growth factor; AR, amphiregulin; RBD, Ras-binding domain; BIM, bisindolylmaleimide; AG1478, tyrphostin; CaM, calmodulin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TGFα, transforming growth factor-α; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; BB94, batimastat

PII: S0014-5793(02)02624-8

against phosphorylated MAPK or phosphorylated MEK were from Cell Signaling, NEBiolabs (Beverly, MA, USA), the monoclonal anti-(pan)-Ras was from Oncogene Sciences (Cambridge, MA, USA); horseradish peroxidase (HRP)-protein A was from Zymed (San Francisco, CA, USA). Peroxidase-labeled antibodies and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight markers were from Bio-Rad. BB94 was from British Biotech (UK). Bisindolylmaleimide (BIM) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were from Calbiochem (Merck Eurolab, Darmstadt, Germany); neutralising antibodies to amphiregulin (AR) (anti-AR, anti-heparin-binding EGF-like growth factor (anti-HB-EGF) and anti-transforming growth factor-α (anti-TGFα) were from R&D Systems Europe (Abingdon, UK). Finally, the anti-phosphotyrosine-RC20 HRP-conjugated was from Transduction Laboratories (Becton Dickinson, San Diego, CA, USA) and the rabbit polyclonal anti-EGFR antibody (Ab1005) from Santa Cruz (Santa Cruz, CA, USA).

2.2. Cell culture

Green monkey kidney cells (COS-1) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), antibiotics and glutamine. DMEM and FCS were purchased from Biological Industries (Beit Haemek, Israel). Cells were grown to about 90% confluence, and starved overnight in those experiments where lysates were used.

2.3. Cellular extracts

After the various treatments (as indicated in figure legends), COS-1 cells (in 35 mm dishes) were lysed in a buffer containing 2% SDS, 67 mM Tris–HCl pH6.8 and 10 mM EDTA, and sonicated twice for 10 s on ice. Equal amounts of protein were electrophoresed and immunoblotted.

2.4. Measurement of Ras activation

The capacity of Ras-GTP to bind to the Ras-binding domain (RBD) of Raf-1 was measured to analyse the amount of active Ras [18]. Cells (2×10^6) were lysed in the culture dish (100 mm) with lysis buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol) plus protease and phosphatase inhibitors. Cleared lysate ($10\,000\times g$) was assayed for protein concentration according to the method of Bradford [19] and protein-equalised supernatants were incubated for 2 h at 4°C with glutathione Sepharose-4B beads pre-coupled to GST-RBD. Beads were washed four times in the lysis buffer. Bound proteins were solubilised by the addition of 30 μl of Laemmli [20] loading buffer and run on 12.5% SDS–PAGE gels. The amount of Ras in the bound fraction was analysed by Western blotting using anti-(pan)-Ras (Ab-3, OP-40, Oncogene Sciences) monoclonal antibody.

3. Results

Recent evidence showed the transactivation of EGFR by phorbol ester (TPA)-dependent activation of PKC [16]. However, since CaM binds and regulates the EGFR signalling we studied the involvement of CaM, as an upstream effector, in the EGFR transactivation and in the overall regulation of the MAPK signalling pathway.

3.1. CaM modulates the PKC transactivation of EGFR

In COS-1 cells, TPA (100 nM), increased the EGFR-tyrosine phosphorylation. This effect was blocked when cells were pre-incubated with Ab225, which prevented the EGF binding site and therefore the extracellular activation of EGFR (Fig. 1A) or after pre-incubation with AG1478 (10 μM), a specific inhibitor of the EGFR tyrosine kinase activity (Fig. 1A).

To examine whether the effect of W-13 was dependent on PKC activity, COS-1 cells were treated with BIM, a PKC inhibitor, before W-13 treatment. Equal amounts of lysates were electrophoresed and the levels of phosphotyrosine EGFR were analysed by Western blot, using the antibody R-C20 HRP-conjugated. Fig. 1B shows that BIM reduces (60%, $n=3$) the effect of W-13 on EGFR tyrosine phosphorylation. This indicates that CaM modulates the transactivation of EGFR by PKC.

Our previous studies showed that W-13-mediated EGFR transactivation requires metalloproteinase activity and subsequent release of growth factors that bind and stimulate the EGFR through an extracellular mechanism [5]. The shedding of different ligands can be promoted by activation of PKC [21,22] or through antagonists of CaM [23]. A battery of neutralising antibodies against some of the most common growth factors was tested. HB-EGF inhibited the W-13-stimulated phosphorylation of EGFR (Fig. 1C); in fact, shedding of HB-EGF was described after PKC activation [24].

These results indicate that transactivation of EGFR, possibly through the release of HB-EGF, by W-13 could be mediated by PKC.

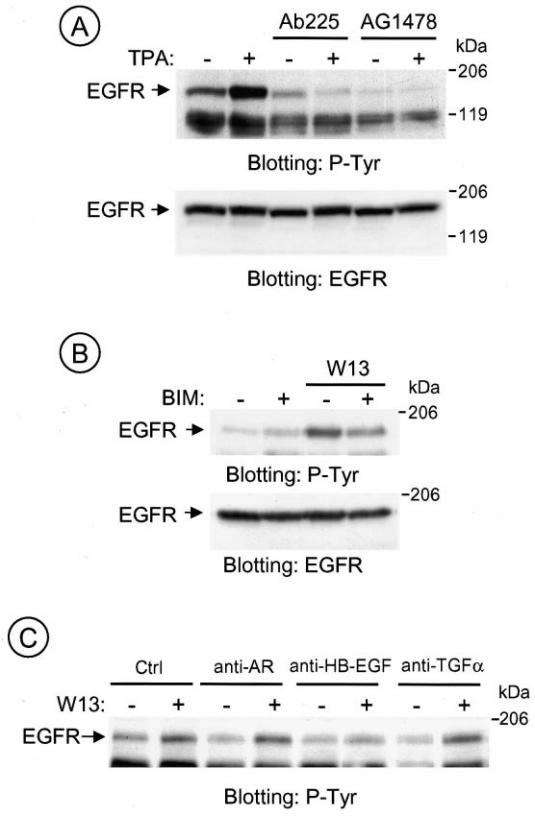

Fig. 1. TPA and W-13 effects on tyrosine phosphorylation of EGFR. A: COS-1 cells were pre-incubated with Ab225 (10 μg/ml) or AG1478 (5 μM) for 15 min, and then treated with TPA (100 nM) for 15 min at 37°C. Equal amounts of protein were electrophoresed and analysed by Western blotting with anti-phosphotyrosine RC-20 antibody HRP-conjugated or with Ab1005, for the EGFR. PKC-mediated EGFR tyrosine phosphorylation is inhibited in the presence of Ab225 or AG1478. B: Treatment with the PKC inhibitor BIM (6 μM) together with W-13 (10 μg/ml) for 30 min. A diminution of W-13-mediated EGFR phosphorylation can be observed when BIM was present. C: The effect of pre-incubation (30 min) of different neutralising antibodies: anti-AR (50 μg/ml), anti-HB-EGF (10 μg/ml) or anti-TGFα (10 μg/ml) on W-13 tyrosine phosphorylation of EGFR was analysed by Western blotting. Only the anti-HB-EGF has an inhibitory effect on the W-13-stimulated phosphorylation of EGFR.

3.2. AG1478 and the metalloproteinase inhibitor BB94 partially block MEK activation by TPA

To ascertain the involvement of CaM in the PKC response we studied the effect of W-13 on EGFR tyrosine phosphorylation by TPA and whether the increase in tyrosine phosphorylation of the EGFR activates the MAPK pathway. The levels of P-MEK were analysed in lysates of COS cells treated with TPA (100 nM) and/or W-13 (10 μg/ml) in the absence or presence of AG1478 or the metalloproteinase inhibitor BB94 (6 μM). Fig. 2A shows that W-13 potentiated (synergistic effect) the TPA effect on EGFR tyrosine phosphorylation. It also shows the activation of MEK by TPA, and that W-13 diminished P-MEK. W-13 acts upstream of MEK inhibiting Raf-1 activity [5] and therefore no synergistic effect of TPA in MEK activation occurred. Interestingly, AG1478 partially reduces the TPA activation of MEK and the subsequent MAPK activation (Fig. 2A).

The same can be observed when the metalloproteinase inhibitor, BB94, was used. Fig. 2B shows that in cells treated with TPA and BB94, P-MEK was not completely inhibited. This result suggests that in our system phorbol esters could activate the MAPK pathway at least by two ways, one dependent on EGFR transactivation and the second independent of the shedding.

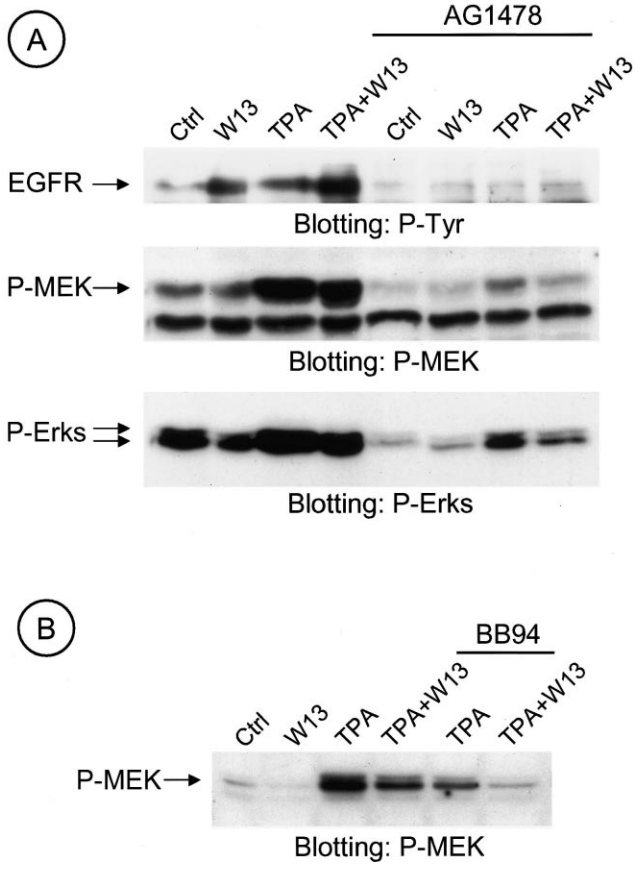

Fig. 2. AG1478 and BB94 partially block MAPK activation by PKC. COS-1 cells were pre-incubated with AG1478 (5 μM) (A) or BB94 (6 μM) (B) for 5 or 15 min at 37°C, respectively. Then, cells were treated with TPA and/or W-13 (15 min). A: Lysates of equal amounts of protein were electrophoresed and phosphotyrosine of EGF-R, MEK activation (P-MEK) or MAPK (P-Erks) were detected by Western blotting with anti-phosphotyrosine RC-20 antibody HRP-conjugated, polyclonal antibody to P-MEK or to P-Erks, respectively. In the absence of AG1478 a synergistic effect can be observed of W-13 and TPA on the tyrosine phosphorylation of EGFR not found on P-MEK or P-Erk. B: Effect of the metalloproteinase inhibitor, BB94, on the P-MEK activated by TPA or TPA+W-13. In both panels (A and B) it can be seen that AG1478 or BB94 treatments do not completely block MEK activation.

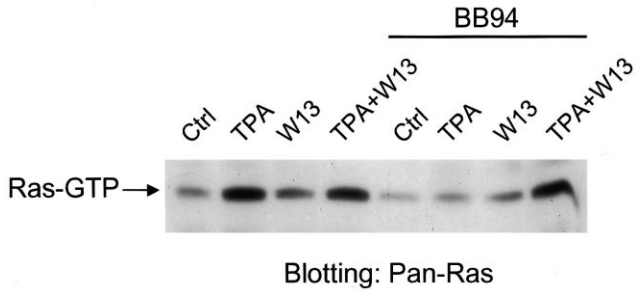

Fig. 3. BB94 does not inhibit Ras activation by TPA in presence of W-13. COS-1 cells grown in 100 mm dishes were incubated with TPA (100 nM) and/or W-13 (10 μg/ml) for 15 min at 37°C and Ras activation (Ras-GTP) was assessed by GST-RBD pull-down, as described in Section 2. Ras-GTP levels were assessed in the presence or absence of the metalloproteinase inhibitor BB94. When metalloproteinases were inhibited (+BB94) it can be observed that incubation of TPA+W-13 restored the amount of activated Ras.

3.3. W-13 facilitates Ras activation by PKC

To study whether CaM modulates the activation of the independent EGFR transactivation of the MAPK cascade by PKC, and to bypass the inhibitory effect of W-13 on the Raf-1 activity, pull-down experiments were performed to analyse the amount of active Ras (Ras-GTP), using GST-RBD recombinant protein. Fig. 3 shows that the metalloproteinase inhibitor BB94 dramatically reduced the levels of Ras-GTP raised by TPA and this inhibition was restored in the presence of W-13. Thus, it can be concluded that CaM is crucial for the PKC-mediated MAPK activation in the EGFR-independent Ras pathway.

4. Discussion

The results of the present study show, in agreement with Chen et al. [16], that PKC is responsible for the stimulation of MAPK through the transactivation of EGFR; in addition, we now demonstrate that this effect can be magnified in the presence of the CaM antagonist W-13, and that CaM exerts an additional effect in the control of the MAPK signalling pathway.

The mechanisms of the PKC-induced EGFR transactivation are complex and involve regulators at different levels. CaM, an intracellular calcium regulatory protein, is directly involved in the proteolytic cleavage of the ectodomain of certain membrane-bound proteins [23]. However, multiple signal transduction pathways can regulate the release of EGFR ligands. Here, in this study we show a synergistic effect of TPA (PKC) and W-13 (inhibition of CaM) in the EGFR transactivation and the subsequent activation of the MAPK signalling pathway.

It has been reported that PKC activation stimulates TGFα, HB-EGF and AR release [25,21]. Experimental evidence points to a possible common mechanism for PKC-regulated release of other cell surface proteins, involving the interaction of metalloproteinases with PKC, as shown for MD9/ADAM9

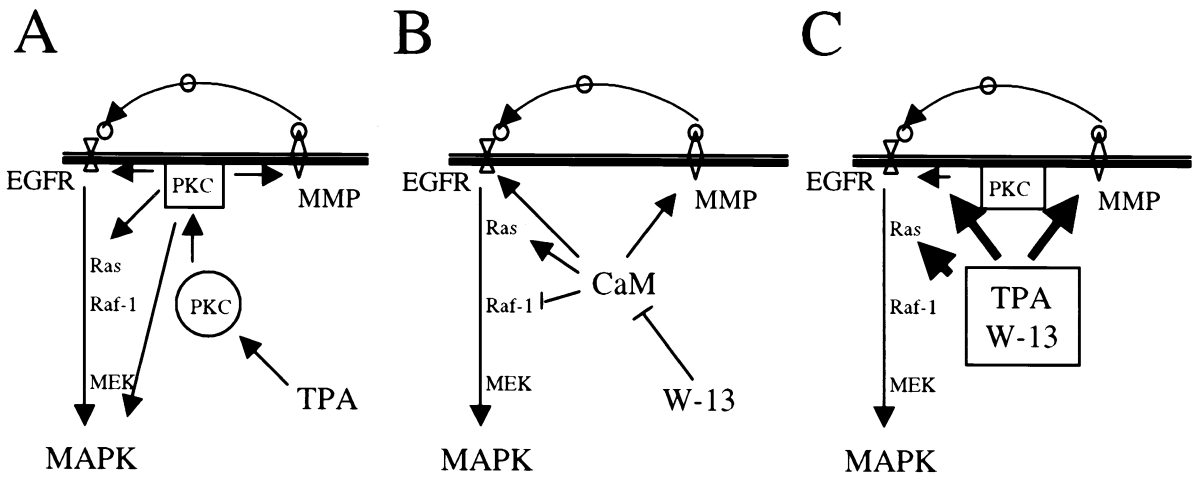

Fig. 4. Effect of TPA and W-13 on the regulation of the EGFR activation and the MAPK signalling pathway in COS-1 cells. A: TPA stimulates, through PKC, the matrix metalloproteinases (MMP), and released growth factors (such as HB-EGF) phosphorylate and activate the EGFR and the subsequent MAPK. Also it is reported that activated PKC phosphorylates EGFR on Thr654 and inactivates its endogenous kinase activity. Moreover, PKC could activate MAPK at different levels. B: The CaM antagonist W-13 mediates the release of growth factors through MMP, activates the tyrosine phosphorylation of EGFR, activates Ras and inhibits Raf-1. C: TPA and W-13 have synergistic effects on the MMP activity, the tyrosine-phosphorylation of EGFR and on the Ras activity. The thin arrow indicates activation, the T-line inhibition and the bold arrow synergistic effects.

and PKCδ in the PMA-induced shedding of HB-EGF in Vero-H cells [24]. However, calcium influx and tyrosine phosphatase activity also regulate the release of EGFR ligands independent of PKC activity [26,27].

When cells were incubated with metalloproteinase inhibitors such as BB94 they became insensitive to the stimulation of EGFR by W-13 [5]. Since recent studies showed that CaM interferes with the shedding of different molecules [28] we used W-13 to find out whether activation of the shedding was dependent on or independent of PKC. In the present study we showed that HB-EGF seems to be involved in the transactivation of EGFR mediated by W-13, since it was shown that incubation with the anti-HB-EGF results in inhibition of the tyrosine phosphorylation of EGFR by W-13.

Regarding the cross-communication between PKC and CaM – in the presence of metalloproteinase inhibitors – for the modulation of Ras-GTP levels and the subsequent MAPK activation, it is conceivable that the direct interaction between Ras and CaM [10] may be responsible for the modulation of PKC on the Ras-GTP levels, by a still unknown mechanism. CaM is the key molecule, which may control the MAPK signalling pathway at different levels; the role of calcium in the activation of CaM is controversial and may differ for different CaM targets. For example, the requirement for calcium is not absolute for CaM binding to L-selectin in vitro [28]. Therefore, location vs. redistribution of CaM by calcium-dependent or -independent mechanisms as well as the differential affinity for CaMBPs, will be decisive for the overall cellular physiology.

Finally, there is the question of where these events take place. Since most signal transduction processes utilise co-localisation of sequentially acting signalling proteins for the selective activation of downstream functions, it is remarkable that in cells treated with W-13 there was an inhibition of the trafficking events leading to the recycling or the degradative pathways [5]; this inhibition of the budding (exit) from aberrant large early endosomal compartment could be responsible for the regulation of MAPK signal transduction. Compartmentalisation (recruitment) of the signal transduction machinery in the endocytic compartment may facilitate the interaction between different 'modules' involved in the regulation of key events that control the activation of MAPK [29–35].

A simplify model which envisage possible intersections between EGFR, PKC and the novel involvement of CaM in the control of the MAPK signalling pathway is shown in Fig. 4. Our data are compatible with a possible general mechanism by which CaM and PKC have an antagonistic effect. For instance, it has been reported that phosphorylation of receptors such as the EGFR [9] or the metabotropic glutamate receptor [36], by PKC prevents its interaction with CaM, and conversely CaM binding inhibits phosphorylation. Other CaMBPs such as MARCKS or adducin seem to be equally regulated.

Acknowledgements: This study was supported by Grant PM99-0166 from the Ministerio de Ciencia y Tecnología, Spain. F.T. is a postdoctoral fellow supported by the Ministerio de Ciencia y Tecnología (Spain). A.Ll. has a fellowship from Fundació Maratò TV3 (99-510). We thank Dr. Richard Marais (Institute of Cancer Research, London, UK) for providing the reagents for the Ras (GTP) pull-down assay.

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