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Elastin peptides activate ERK1/2 via a Ras-independent mechanism requiring both p110g/Raf-1 and PKA/B-Raf signaling in human skin fibroblasts

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RUNNING TITLE

Mechanism of elastin peptide-dependent ERK1/2 activation

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ABBREVIATIONS : βARK1-CT, C-terminal domain of the β-adrenergic receptor kinase 1; EGF, Epidermal Growth Factor; DMEM, Dulbecco's Modified Eagle Medium; EBP, elastin binding protein; ERK1/2, extracellular-signal regulated kinase 1/2; GST, glutathione S-transferase; H-89, *N*-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide; LY294002, 2-(4-morpholynil)-8-phenyl-4H-1-bemzopyran-4-one; LY303511, 2-Piperazinyl-8-phenyl-4H-1-benzopyran-4-one MEK1/2, mitogen-activated protein kinase/extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3 kinase; PI-3P, phosphatidylinositol-3-phosphate.

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ABSTRACT

Elastin peptides (EP) produced during cancer progression bind to the elastin binding protein (EBP) found at the surface of dermal fibroblasts, leading to the expression of collagenase-1 gene. The production of this enzyme involved in stromal reaction is due to the sustained activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway via cAMP/protein kinase A (PKA) and phosphatidylinositol-3 kinase (PI3K). However, the mechanism of these signaling events remains unknown. We show that kappa-elastin (kE), a commonly used EP, induces maximum phosphorylation of MEK1/2 and ERK1/2 after 30 min. The simultaneous inhibition of PKA and PI3K, by *N*-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H-89) and 2-(4-morpholynil)-8-phenyl-4H-1-bemzopyran-4-one (LY294002) respectively, blocked MEK1/2 and ERK1/2 phosphorylation, as did lactose, an EBP antagonist. kE induced Raf-1 phosphorylation and activation in a PI3K-dependent manner. In our system, the PI3K p110γ is expressed and activated by βγ-derived subunits from a pertussis toxin-sensitive G protein following fibroblast stimulation. Pertussis toxin also blocks the Raf-1/MEK1/2/ERK1/2 phosphorylation cascade. Additionally, we found that B-Raf is expressed in dermal fibroblasts and activated in a PKA-dependent manner following kE treatment, thereby integrating PKA signals to MEK1/2. Importantly, Ras involvement was excluded as ERK1/2 activation by kE was not blocked in RasN17-transfected fibroblasts. Collectively, our results identify a novel Ras-independent ERK1/2 activation system in which p110γ/Raf-1/MEK1/2 and PKA/B-Raf/MEK1/2 cooperate to activate ERK1/2. Thus, p110γ and B-Raf appear as important modulators of dermal fibroblasts physiology and should now qualify as therapeutic targets in strategies aiming at limiting elastin degradation contribution to cancer progression.

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INTRODUCTION

Elastin is the extracellular matrix molecule responsible for resilience of tissues and has been firstly thought to be restricted to that role. It is now established that elastin degradation may lead to the production of bioactive peptides (Hornebeck et al., 2002) influencing cell chemotaxis, cell proliferation and proteases synthesis in a broad panel of normal and tumor cells (Duca et al., 2004). Their contribution to the stromal reaction is therefore envisaged (Hornebeck et al., 2002).

The receptor of these peptides comprises three subunits. The first two, a 55-kDa cathepsin A termed protective protein (EC 3.4.16.1) and a 61-kDa neuraminidase (EC 3.2.1.18), are membrane-associated. The last subunit, which actually binds elastin-derived peptides (EP), is a peripheral 67-kDa protein, termed elastin binding protein (EBP). This protein possesses galactolectin properties. When galactosugars bind EBP, its affinity for EP dramatically decreases, leading to their release and to further dissociation of EBP from the complex. Galactosugars, such as lactose, are therefore commonly used as EBP antagonists (Hinek, 1996).

In human dermal fibroblasts, an elastin degradation product, κ-elastin (kE), up-regulates the expression of pro-collagenase-1, whom activated form is crucially involved in stromal reaction (Brassart et al., 2001). We have recently shown (Duca et al., 2002) that the extracellular-signal regulated kinase 1/2 pathway (ERK1/2) holds a central role in the signaling leading to this phenomenon. In our system, ERK1/2 activation involved both cAMP-dependent activation of protein kinase A (PKA) and phosphatidylinositol-3 kinase (PI3K). However, the details of this integrated cross-talk were not elucidated.

ERK1/2 activation requires their phosphorylation by dual specific kinases named mitogen-activated protein kinase/extracellular signal-regulated kinases 1 and 2 (MEK1/2), which are

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themselves typically phosphorylated and activated by kinases named Raf (Houslay and Kolch, 2000). In mammals, the Raf family comprises three members : the ubiquitously expressed Raf-1, A-Raf and B-Raf whom expressions are more restricted. Compared to Raf-1, B-Raf is a stronger activator of the ERK pathway while A-Raf is weaker (Chong et al., 2003; Mercer and Pritchard, 2003). Raf-1 is activated by the small GTP-binding protein Ras but other activators such as protein kinase C (PKC) or PI3K have also been reported (Dhillon and Kolch, 2002). The phosphorylation of Raf-1 on S338 is essential for its activation (Mason et al., 1999).

Three classes of PI3K have been characterized (Anderson and Jackson, 2003), but only those belonging to class I, comprising subclasses I_A and I_B, have been shown to activate ERK1/2. This activation occurs at the level of Raf-1 or MEK1/2 (King et al., 1997; Mas et al., 2003; Takeda et al., 1999).

Class I_A PI3K present a p85 regulatory subunit associated to a p110α, p110β or p110δ catalytic subunit. They are activated by phosphotyrosine motifs and/or Ras. Class I_B ones possess a p101 regulatory subunit associated to a p110γ catalytic subunit, which can be directly activated by G protein βγ subunits or Ras (Anderson and Jackson, 2003). Class I_A PI3K are expressed in fibroblasts (Anderson and Jackson, 2003), but the presence of p110γ has not been reported yet.

B-Raf is expressed as multiple alternatively spliced variants but possesses two major isoforms : 68 and 95 kDa (Mercer and Pritchard, 2003). It is an important regulator of ERK1/2 pathway activation by cAMP-dependent signaling elements (Houslay and Kolch, 2000). cAMP activates ERK1/2 cascade in cells expressing the 95 kDa isoform while it is inhibited in NIH 3T3 fibroblasts lacking it (Vossler et al., 1997). cAMP-dependent activation of the 68 kDa isoform was reported suggesting that it could participate to cAMP-dependent ERK1/2 pathway induction (Seidel et al., 1999). cAMP-dependent B-Raf activation could

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occur via two mechanisms involving either the cAMP receptor Epac (exchange protein directly activated by cAMP) or the cAMP-dependent protein kinase PKA (Houslay and Kolch, 2000). Although B-Raf is expressed in mouse embryonic fibroblasts and seems to be absent in NIH3T3 fibroblasts (Huser et al., 2001; Vossler et al., 1997), its presence in human skin fibroblasts has not been reported.

We describe here the mechanisms leading to ERK1/2 activation by elastin-peptides in dermal fibroblasts. We show that kE activates ERK1/2 via both p110γ/Raf-1/MEK1/2 and PKA/B-Raf/MEK1/2 modules. Such a Ras-independent signaling system is novel and provides a new regulation system for ERK1/2 activation. Additionally, we show that p110γ and B-Raf, which had never been reported in dermal fibroblasts are important modulators of the physiology of these cells. As their involvement could explain the strong and sustained activation of ERK1/2 and subsequent pro-collagenase-1 production, their control in strategies aiming at limiting elastin peptides contribution to cancer progression is discussed.

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MATERIALS AND METHODS

Materials. kE was prepared as previously described (Brassart et al., 2001). Human recombinant epidermal growth factor (EGF) was from Upstate (distributed by Euromedex, Mundelsheim, France). Lactose, pertussis toxin (PTX), phosphatidylserine, phosphatidylinositol, phosphatidylinositol-3-phosphate and proteases inhibitors cocktail (Ref. P8340) were from Sigma (Saint-Quentin Fallavier, France). H-89, *i.e.* *N*-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide, KT5720 and LY303511 *i.e.* 2-piperazinyl-8-phenyl-4H-1-benzopyran-4-one were from Calbiochem (distributed by VWR Int., Strasbourg, France). LY294002, *i.e.* 2-(4-morpholynil)-8-phenyl-4H-1-benzopyran-4-one, was purchased from Cell Signaling Technology (distributed by Ozyme, Saint-Quentin en Yvelines, France). Recombinant GST-MEK1 was from Santa-Cruz Biotechnology (distributed by Tebu, Le Perray en Yvelines, France). Mouse monoclonal anti-β-actin antibody was purchased from Sigma. Rabbit polyclonal phospho-specific antibodies against active forms of ERK1/2 (phosphorylated on T202 and Y204), MEK1/2 (phosphorylated on S217 and S221) and phospho-specific antibody against phosphorylated Raf-1 S338 and anti-ERK1/2 antibody were from Cell Signaling Technology. Rabbit anti-p110γ, anti-Raf-1 and anti-B-Raf polyclonal antibodies were purchased from Santa-Cruz Biotechnology. Polyclonal anti-p85 antibody was from Upstate Biotechnology Inc. (distributed by Euromedex, Mundolsheim, France). Mouse monoclonal anti-HA tag was from Roche Molecular Biochemicals (Meylan, France). All reagents for cell culture and transfection reagent LipofectAMINE 2000 were from Invitrogen (Cergy Pontoise, France). Enhanced chemiluminescence substrate kit and [γ-^{32}P]-ATP were purchased from Amersham Biosciences (Orsay, France). Others reagents were from Sigma.

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Expression Plasmids. The plasmid pRK5-βARK1-CT (Koch et al., 1994), which encodes the G495-L689 fragment corresponding to the C-terminus of β-adrenergic receptor kinase 1 (βARK1-CT), was kindly provided by Dr. R.J. Lefkowitz (Durham, USA). The constructs encoding HA-tagged-ERK1 (pECE-HA-ERK1) and the dominant negative RasN17 mutant (pSV-RasN17) were previously described (Guillemot et al., 2000), and were kind gifts from Dr. J. Pouysségur (Nice, France) and Dr. F. Schweighoffer (Chatenay-Malabry, France), respectively.

Cell culture, treatments and transfection. Human skin fibroblast strains were established from explants of human adult skin biopsies obtained from informed healthy volunteers (age 21-49 years). Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2mM glutamine in the presence of 5% CO_2. Cells at subcultures 5 to 10 were used. For experiments, fibroblasts were grown to subconfluency in 10% serum containing medium. Before stimulation, cells were incubated for 18 h in DMEM containing 0.5 % fetal calf serum, washed twice with PBS, then incubated in serum-free DMEM with or without kE (50 µg/ml) or EGF (2 ng/ml) for the indicated times. The pharmacological inhibitors H-89 (1 µM), KT5720 (2 µM), LY294002 (25 µM), and LY303511 (25 µM) were preincubated 1h before stimulation, whereas lactose (1 mM) and PTX (100 ng/ml) were preincubated 3 h and 18 h respectively. Elastin peptide stimulation was stopped by adding ice-cold PBS containing 50 µM Na_3VO_4. For transfection experiments, cells were grown to 80-90% of confluency in 10% serum containing medium, and incubated 16 h in serum-free DMEM with LipofectAMINE 2000-DNA plasmid complexes. The ratio was 2 µl of LipofectAMINE 2000 for 2 µg total plasmidic DNA for 5×10^5 cells. Growth medium was then added for 24 h and cells were treated before stimulation as described above.

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Western-blotting. Cells (10^6) were washed twice in ice-cold PBS containing 50 µM Na_3VO_4, scrapped and sonicated in lysis buffer (PBS pH 7.4, 0.5 % Triton X-100, 80 mM β-glycerophosphate, 50 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, proteases inhibitors cocktail). Insoluble material was removed by centrifugation (20 000 *g* / 20 min / 4°C). Protein concentrations were determined by BCA protein assay (Pierce, distributed by Interchim, Montluçon, France). Equal amounts of proteins were heated for 5 min at 100°C in Laemmli sample buffer, resolved by SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes. The membranes were placed in blocking buffer [5 % (w/v) non-fat dry milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % (v/v) Tween 20)] for 1 h at room temperature and incubated overnight at 4°C with anti-phospho-ERK1/2 (1:1000), anti-phospho-MEK1/2 (1:1000), anti-phospho-S338-Raf-1 (1:1000), anti-B-Raf (1:500), anti-p110γ (1:500), anti-ERK1/2 (1:1000) or anti-β-actin (1:5000) antibodies. After five washings with TBST, the membranes were incubated for 1 h at room temperature in the presence of horseradish peroxydase-coupled anti-rabbit or anti-mouse antibodies (1:4000 and 1:10000 in blocking buffer, respectively). Immunocomplexes were detected by chemiluminescence. Blots were semi-quantified by densitometry using PhosphorAnalyst software (Bio-Rad, Marne-la-Vallée, France).

Immunoprecipitation and PI3K activity assay. Cells (8×10^6) were washed twice in ice-cold PBS containing 50 µM Na_3VO_4, scrapped in this buffer and centrifuged (375 *g* / 10 min / 4°C). Pellets were resuspended and lysed for 15 min at 4°C in immunoprecipitation lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Brij 98, 1mM Na_3VO_4, proteases inhibitor cocktail). Insoluble material was removed by centrifugation (20 000 *g* / 20 min / 4°C). Protein concentrations were determined using BCA protein assay and equal amounts of proteins were incubated with 2.5 µg of anti-p85 or anti-

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p110γ antibodies for 1 h at 4°C. The antigen-antibody complexes were incubated with protein G-Plus-Sepharose (Santa Cruz Biotechnology) for 1h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer then twice with lipid kinase buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 200 µM adenosine). To perform lipid kinase assay each pellet was resuspended in 70 µl of lipid kinase buffer supplemented with phosphatidylinositol and phosphatidylserine (10 mg/ml each), 2.5 µM ATP and 10 µCi [γ-^{32}P]-ATP. The reaction was performed for 15 min at 30°C and stopped by adding 100 µl of 1M HCl. Phospholipids were extracted with 350 µl of chloroform/methanol (1:1, v/v), and the organic phase was washed twice with 200 µl of methanol/1M HCl (1:1, v/v). Organic phases (110 µl) were spotted on to oxalate-treated thin layer chromatography (TLC) plates and lipids were then separated using a chloroform/methanol/acetone/acetic acid/H_2O (40:13:15:12:7, v/v/v/v/v) solvent system. Plates were revealed by autoradiography. For transfection experiments, cells were transfected with either pRK5-βARK1-CT or the corresponding empty vector before kE treatment (2 µg of plasmid DNA for 0.5×10^6 cells).

Raf-1 and B-Raf activities assay. Samples preparation and immunoprecipitation were performed as described above. Equal amounts of proteins were incubated with 2.5 µg of anti-Raf-1 or anti-B-Raf antibodies for 1 h at 4°C. The antigen-antibody complexes were incubated with protein G-Plus-Sepharose for 1h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer and twice with kinase buffer (20 mM Hepes pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$). For kinases activity assays, pellets were resuspended in 50 µl kinase buffer supplemented with 1 µg GST-MEK1, 2.5 µM ATP and 10 µCi [γ-^{32}P]-ATP. The reaction was performed for 30 min at 30°C and stopped by adding Laemmli sample buffer. After boiling at 100°C for 5 min, samples were resolved on a 10% SDS-PAGE, gels were dried and bands were visualized by autoradiography.

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Assessment of HA-tagged-ERK1 activation. Cells (10^6) were cotransfected with 2 µg of pECE-HA-ERK1 and 2 µg of pSV-RasN17, or with the corresponding empty vectors prior to stimulation. Equal amounts of proteins were incubated for 1h at 4°C with 3 µg of anti-HA tag antibody. The formed antigen-antibody complexes were incubated with protein G-Plus-Sepharose for 1h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer then resuspended in Laemmli sample buffer. Proteins extracts were then resolved on 10% SDS-PAGE and active phospho-HA-tagged-ERK1 and HA-tagged-ERK1 were visualized using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies.

Statistical Analysis. All experiments were performed in triplicate. Results are expressed as mean ± S.E.M. Comparison between groups were made using Student's t test. The results were considered significantly different at $p<0.05$.

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RESULTS

kE induces MEK 1/2 activation via PI3K and PKA-dependent signaling.

In a previous work, we have shown that, the treatment of human skin fibroblasts with kE was followed by a rise of the intracellular cAMP level and that PKA activation participated to ERK1/2 induction. Forskolin alone partly reproduced the effect of kE, demonstrating that cAMP-dependent signaling was not sufficient to achieve maximum induction of the ERK1/2 pathway. Indeed, PI3K participation was required to fully activate ERK1/2 (Duca et al., 2002). Nevertheless, the molecular links between ERK1/2 and their upstream activators were not revealed.

MEK1/2 activates ERK1/2. We therefore analyzed its phosphorylation pattern by Western-blot using an antibody specifically recognizing its active form *i.e.* phospho-S217/S221-MEK1/2. In parallel, the presence of the phospho-T202/Y204-ERK1/2 active form was checked.

The treatment of fibroblasts with 50 µg kE/ml resulted in a sustained activation of MEK1/2 up to a maximum reached at 30 min of stimulation (Fig. 1A). The time-course of this activation closely paralleled that observed for ERK1/2 (Fig. 1A). Interestingly, a strong activation of both kinases still persists after 60 minutes of stimulation.

The importance of PKA and PI3K signaling towards MEK1/2 activation was investigated using pharmacological inhibitors. When cells were pre-treated for 1 h with H-89 (1 µM) or LY294002 (25 µM), two commonly used PKA and PI3K inhibitors respectively, MEK1/2 and ERK1/2 activations were partially blocked (Fig. 1B). However, their simultaneous use totally abolished MEK1/2 and ERK1/2 activation (Fig. 1B). The same effect was obtained using 1 mM lactose (Fig. 1B).

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In order to support these observations, kE-induced ERK1/2 activation was assessed in the presence of LY303511 (25 µM), a compound that is structurally similar to LY294002 but does not inhibit PI3K. In addition, we inhibited PKA with KT5720 (2 µM), a PKA inhibitor structurally unrelated to H-89. Our results show (Fig. 1C) that LY303511 has no effect on kE-induced ERK1/2 activation, whereas the use of KT5720 reproduced the effect of H-89, supporting our previous observations.

PKA and PI3K thus appeared to be crucial modulators of the MEK1/2/ERK1/2 cascade in elastin peptides-induced signaling.

Inhibition of PI3K blocks kE-induced Raf-1 S338 phosphorylation and Raf-1 activation.

Raf-1 is the typical activator of MEK1/2 (Houslay and Kolch, 2000), the immediate upstream activator of ERK1/2. Its activation is mainly related to its phosphorylation on S338 (Mason et al., 1999). Several authors have suggested that S338 phosphorylation and Raf-1 activation could be regulated by PI3K (Chaudhary et al., 2000; Sun et al., 2000). For these reasons, we examined the effect of kE on S338 phosphorylation and on Raf-1 activity in presence of LY294002. Using an anti-phospho-S338-Raf-1 antibody, we show by Western-blot that kE cell stimulation leads to S338 Raf-1 phosphorylation (Fig. 2A) with a time-course very similar to those observed for MEK1/2 and ERK1/2 (Fig. 1A). The maximum phosphorylation level was observed at 30 min. The inhibition of PI3K with LY294002 (25µM) totally blocked S338 phosphorylation induced by kE treatment (Fig. 2B). However, even if phosphorylation of S338 is required for Raf-1 activation, it is not a surrogate marker for Raf-1 activity (Chiloeches et al., 2001; Mason et al., 1999). We therefore analyzed Raf-1 activity under the same conditions. Using GST-MEK-1 as a substrate, we found that Raf-1 activity was very low in resting skin fibroblasts (Fig. 2C, first lane). However, when cells

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were treated with kE, its activity was raised five folds (Fig. 2C, second lane). Pretreatment of cells with LY294002 totally blocked Raf-1 activity. PKA inhibition (1 µM H-89) had no effect on Raf-1 activity (data not shown).

These results indicated that kE induced Raf-1 S338 phosphorylation and its activation via PI3K and that PI3K integrated the MEK1/2/ERK1/2 pathway at the level of Raf-1. They also suggested that PKA signaling should involve a different integration system.

kE promotes class I_B PI3K activation in human skin fibroblasts.

Both class I_A and class I_B PI3K were shown to be involved in ERK1/2 activation in various cell types (King et al., 1997; Mas et al., 2003; Takeda et al., 1999). PI3K activity of these two subfamilies was therefore analyzed following kE cell stimulation.

Class I_A PI3K associate with a regulatory p85 subunit. They are found in fibroblasts (Anderson and Jackson, 2003). In contrast, p110γ binds a p101 regulatory subunit and its tissue distribution is more limited (Yart et al., 2002). It is highly expressed in haematopoietic cells but its presence has also been reported in non-haematopoietic cells, notably melanoma cells (Lee et al., 2002). To our knowledge, its expression has never been reported in skin fibroblasts.

First, total cells extracts were Western-blotted with a specific anti-p110γ antibody showing that this PI3K isoform is expressed in human skin fibroblasts (Fig. 3A). We then measured PI3K activity in p85 and p110γ immunoprecipitates using an *in vitro* PI3K activity assay with phosphatidylinositol (PI) as a substrate. We found (Fig. 3B) that kE treatment led to an important augmentation of PI3K activity in p110γ immunoprecipitates (about 150% increase compared to control). A non-significant increase was observed in p85 immunoprecipitates.

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These results show that class I_B PI3K, p110γ, is expressed in human skin fibroblasts and that its activity is strongly stimulated by elastin peptides. Additionally, Figure 3C shows that, in our experimental conditions, LY294002 inhibited p110γ whereas LY303511 had no effect. These results are in agreement to those observed for the inhibition of ERK1/2 activation under the same conditions (Fig. 1B and 1C).

p110g is activated by b g subunits of pertussis toxin-sensitive G protein.

Several authors have suggested that EBP could be coupled to a PTX-sensitive G protein (Brassart et al., 2001; Mochizuki et al., 2002). As the ERK1/2 pathway and p110γ activation can be due to such a G protein (Takeda et al., 1999; Yart et al., 2002), we therefore analyzed the effects of PTX treatment on ERK1/2 pathway and p110γ activation following elastin peptides stimulation.

Pretreatment with PTX (100 ng/ml, 18h) totally blocked Raf-1 S338 phosphorylation while MEK1/2 S217/221 and ERK1/2 T202/Y204 phosphorylations were partially inhibited (Fig. 4A). As these observations were similar to those obtained with PI3K blocking (Fig. 1B, and 2B), they strongly suggested that p110γ was activated by such a G protein. We thus checked the effect of PTX on p110γ activity. As expected, pretreatment of cells with PTX (100 ng/ml, 18h) returned kE-induced p110γ activity to control level (Fig. 4B). These data show that a PTX-sensitive G protein is required for p110γ activation.

Initially, when p110γ was cloned, it was reported that its activity could be regulated *in vitro* by either α or βγ G protein subunits (Stoyanov et al., 1995). However, further *in vivo* studies pointed out the crucial role of the βγ heterodimer (Brock et al., 2003). So, we investigated the possible role of βγ subunits in p110γ activation.

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To test this hypothesis, human skin fibroblasts were transiently transfected with a plasmid encoding the C-terminal domain of the β-adrenergic receptor kinase 1 (βARK1-CT) which acts as a βγ-scavenger molecule (Koch et al., 1994). The expression of the βARK1-CT polypeptide significantly inhibited kE-induced p110γ activation (Fig. 4C), showing that βγ subunits from PTX-sensitive G proteins are required for p110γ activation. It is important to underline here that the inhibition level reported in Figure 4C only reflects the inhibition of this kinase in pRK5-βARK1-CT-transfected fibroblasts.

Taken together, our data show that the treatment of human skin fibroblasts with kE triggers a PTX-sensitive G protein whom βγ subunits activate p110γ.

B-Raf is expressed in skin fibroblasts and is activated by elastin-peptides in a PKA dependent-manner.

B-Raf has been shown to be an upstream activator of MEK1/2 and has the capacity to positively integrate cAMP signals to the ERK1/2 pathway (Houslay and Kolch, 2000). Its expression pattern is restricted compared to Raf-1 (Mercer and Pritchard, 2003), but, although its presence was reported in mouse embryonic fibroblasts (Huser et al., 2001), it is absent in NIH3T3 fibroblasts (Vossler et al., 1997). To our knowledge, its expression has never been reported in human skin fibroblasts.

The Western-blot analysis of B-Raf expression in these cells (Fig. 5A) revealed that they expressed its 95 kDa isoform whereas the 68 kDa isoform was apparently absent. As B-Raf can positively integrate cAMP signaling to ERK1/2 activation in a PKA-dependent manner (Schmitt and Stork, 2002), we analyzed B-Raf kinase activity, using GST-MEK1 as a substrate, in the presence of H-89.

In resting cells, B-Raf activity was weak (Fig. 5B, lane 1) but strongly increased (5 folds) following kE treatment (Fig. 5B, lane 2). The addition of H-89 (1 µM, pretreatment for

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1h) totally blocked B-Raf activation (Fig. 5B, lane 3). The involvement of PKA in B-Raf activation was further confirmed using another PKA inhibitor, KT5720 (Fig. 5C).

These findings show that B-Raf is expressed in human skin fibroblasts and that it is activated in the presence of elastin peptides. Its activation is PKA-dependent and leads to MEK1/2 and subsequent ERK1/2 phosphorylation.

kE-triggered ERK activation is independent of Ras.

The small G protein Ras is a major activator of the ERK1/2 pathway. It can activate Raf-1, B-Raf and p110γ (Chong et al., 2003; Mercer and Pritchard, 2003; Suire et al., 2002).

In order to evaluate the contribution of Ras in kE-induced ERK1/2 activation, skin fibroblasts were transiently cotransfected with HA-tagged-ERK1 and a dominant-negative Ras, RasN17, and further stimulated with kE for 30 min.

After HA-immunoprecipitation, ERK1 phosphorylation was assessed by Western-blot using the antibody recognizing the active forms of ERK1/2. The analysis of HA-tagged-ERK1 phosphorylation revealed an increase in ERK1 phosphorylation in stimulated cells (Fig. 6A, lane 3). This result was consistent with previous data (Fig. 1A). The over-expression of RasN17 did not block ERK1 phosphorylation in kE-stimulated cells (Fig. 6A, lane 4). A non-significant increase in ERK1 phosphorylation was observed when RasN17-transfected cells were stimulated with kE. The inhibitory effect of RasN17 expression on Ras-dependent ERK1/2 activation in our cells was assessed by monitoring ERK1/2 activation in RasN17-transfected human dermal fibroblasts after EGF treatment (Fig. 6B, lane 4 compared to lane 3).

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These results show that the activation of the ERK pathway observed after treatment of human skin fibroblasts with kE is independent of Ras.

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DISCUSSION

The *in vivo* generation of EP is thought to influence cancer progression (Hornebeck et al., 2002). Fibroblasts play a fundamental role during the stromal reaction (Westermarck and Kahari, 1999) and because they respond to EP presence by secreting pro-collagenase-1 (Brassart et al., 2001), their contribution to metastasis development could be accentuated in such conditions.

In skin fibroblasts, kE up-regulate collagenase-1 expression through PKA, PI3K and ERK1/2 pathway activation (Duca et al., 2002). However, the detailed mechanisms of these PKA- and PI3K-signalings were unexplained. We show here that kE activates ERK1/2 via a Ras-independent mechanism involving p110γ/Raf-1/MEK1/2 and PKA/B-Raf/MEK1/2 cascades.

Up to now, the activation of ERK1/2 by EP has been reported in smooth muscle cells (SMC) (Mochizuki et al., 2002), fibroblasts (Duca et al., 2002) and monocytes (Fulop et al., 2001). However, the activation of MEK1/2, the immediate upstream activator of ERK1/2, had not been reported. We show here that kE treatment leads to the phosphorylation of MEK1/2 on S217/221, thereby generating their active forms. This activation cascade occurs in PI3K- and PKA-dependent manners (Fig. 1). This observation is consistent with the previously described induction of ERK1/2 phosphorylation by these matrix peptides (Duca et al., 2002). As the simultaneous inhibition of PI3K and PKA totally blocked MEK1/2 and ERK1/2 activation, reproducing the effect of lactose, we concluded that those enzymes were crucial components in EP-induced MEK1/2 and ERK1/2 activation in skin fibroblasts.

In our system, the phosphorylation of Raf-1 on S338 and its kinase activity are controlled by PI3K activity and lead to MEK1/2 activation (Fig. 2).

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The possible involvement of Raf-1 in elastin signaling had been suggested as the missing link between Ras and MEK1/2 in porcine SMC (Mochizuki et al., 2002). Indeed, these authors used radicicol which acts as a depleting agent for Raf-1 (Soga et al., 1998). In contrast, our work demonstrates the activation of Raf-1 following stimulation of cells by EP.

It is well established that phosphorylation of Raf-1 on S338 is essential for its activation (Mason et al., 1999). The contribution of PI3K to these events has been proposed (Chaudhary et al., 2000; Sun et al., 2000). Nevertheless, this model was challenged (Chiloeches et al., 2001). The authors showed that LY294002 inhibited Raf-1 S338 phosphorylation only at concentrations far greater than those required to inhibit PI3K. They thus suggested that Raf-1 S338 phosphorylation inhibition could not be attributed to PI3K.

Here, we blocked S338 phosphorylation and Raf-1 activity using LY294002 (Fig. 2), at a commonly used and rather low concentration (25 µM). Our data thus suggest that, in fibroblasts, S338 phosphorylation could be PI3K-dependent, which supports the hypothesis that PI3K could contribute to Raf-1 S338 phosphorylation and activation (Chaudhary et al., 2000; Sun et al., 2000).

We analyzed the effects of kE stimulation on the activities of class I PI3K. In dermal fibroblasts, the activity of class I_A isoforms remained unchanged after kE treatment. In contrast, p110γ activity was strongly increased (Fig. 3). This finding shows, for the first time, that human skin fibroblasts do possess an efficient p110γ.

We tested the effect of PTX on Raf-1/MEK/ERK cascade and found that PTX blocked Raf-1 S338, MEK1/2 and ERK1/2 phosphorylation with an efficiency comparable to that observed using LY294002. This parallel between the results observed with PTX and those related to PI3K inhibition (Fig. 4A) prompted us to propose that a G protein-inducible PI3K closed the gap between G protein and Raf-1 activation. Our data suggest that p110γ could be

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that kinase. This proposal is strongly supported by the fact that PTX totally blocked p110γ activity (Fig. 4B).

A class I_A PI3K, p110β, has been shown to be stimulated by G βγ subunits (Murga et al., 2000). Nevertheless, this process requires its pre-activation by binding of its p85 regulatory subunit to phospho-tyrosine motifs (Yart et al., 2002). In this case, βγ act as p110β activity enhancers and, consequently, the blocking of these subunits partly inhibits the activation of p110β downstream targets. The very weak PI3K activity we observed in p85 immunoprecipitates (Fig. 3B) is non significant. So, the participation of p110β to ERK1/2 activation was excluded. Additionally, Raf-1 S338 phosphorylation was fully inhibited by PTX (Fig. 4A), indicating that the activation of a G protein was sufficient to transduce the signal to Raf-1.

βγ heterodimers are important regulators of p110γ *in vivo* (Brock et al., 2003). We observed that transient transfection of dermal fibroblasts with a construct encoding the βARK1-CT polypeptide blocked p110γ activity (Fig. 4C). Our results are consistent with data showing βγ requirement for p110γ activation (Brock et al., 2003).

Collectively, our observations strongly suggest that kE-mediated ERK1/2 activation by PI3K could be attributed to p110γ.

We have shown that kE stimulation of fibroblasts increased the intracellular level of cAMP, thereby inducing PKA activation and its participation to ERK1/2 activation (Duca et al., 2002). However, the mechanisms involved were not described.

PKA can participate to ERK1/2 pathway activation via B-Raf activation but the occurrence of this mechanism had never been reported in dermal fibroblasts. Our work constitutes the first observation of B-Raf expression in dermal fibroblasts. The 95 kDa isoform was detected, whereas its 68 kDa counterpart was apparently absent (Fig. 5A). B-Raf

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kinase activity on MEK was strongly increased by kE and could be totally blocked using H-89 or KT5720 (Fig. 5B and 5C). Thus, B-Raf activation appeared PKA-dependent. To our knowledge, this is the first time that a system describing MEK induction via PKA-dependent activation of B-Raf is reported in dermal fibroblasts. B-Raf activation by cAMP via PKA-dependent mechanism involves the small G protein Rap-1 (Houslay and Kolch, 2000). It would be interesting to explore its role in our system.

We point out here that the mechanism leading to the cAMP increase remains unknown and should also be explored. Indeed, PTX-sensitive G protein βγ subunits can activate the adenylyl cyclase (Albert and Robillard, 2002). However, such a mechanism can not be involved in our system because PTX failed to totally block EP-induced signaling.

The use of PTX has already permitted to demonstrate the involvement of a G_i/G_0 in EP signaling (Brassart et al., 2001; Mochizuki et al., 2002). Our work support this view but raises the intriguing possibility that a G_s could also participate. The dual activation of G_i and G_s by the same receptor has been reported (Herrlich et al., 1996; Zou et al., 1999). In our system, this point should deserve specific attention because the mechanisms leading to G protein activation by the elastin receptor remain largely unknown, a fact mostly due to our ignorance of its operational mechanism.

It was suggested that EP could lead to Ras induction in porcine SMC (Mochizuki et al., 2002). Moreover, Ras can directly activate Raf-1, B-Raf and p110γ (Mercer and Pritchard, 2003; Suire et al., 2002). For these reasons, we evaluated Ras contribution to ERK pathway activation. Our results with dermal fibroblasts transfected with the dominant negative RasN17 mutant strongly suggest that the kE-induced ERK activation does not require Ras (Fig. 6A). The slight increase in HA-tagged-ERK-1 phosphorylation we observed when kE-treated fibroblasts were transfected with RasN17 was non significant. A comparable, but more

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important effect, was reported for nocodazol-treated RasN17-transfected HEK293T cells (Zang et al., 2001). This intriguing phenomenon is not explained.

The dual activation of Raf isoforms and their complementary contribution to ERK1/2 activation have been described in other cells (Garcia et al., 2001; Norum et al., 2003), but examples of its occurrence remain scarce. We provide another example of this type of regulation and identify dermal fibroblasts as B-Raf expressing cells. Being the strongest ERK1/2 activator within the Raf family (Mercer and Pritchard, 2003), B-Raf should be considered as a crucial ERK1/2 activator in these cells. We emphasize that the concerted action of Raf-1 and B-Raf could explain the sustained ERK1/2 activation observed in kE-treated fibroblasts, as shown for thrombopoietin-stimulated UT7-Mpl cells (Garcia et al., 2001).

We also establish that p110γ is expressed in dermal fibroblasts and strongly activated by kE. EP are well known for their chemotactic activity and the migration they promote. p110γ is a critical element in the development of the chemotactic response in neutrophils (Hirsch et al., 2000). We think that this enzyme could be a key element of EP-induced migration.

EP are produced in normal and physiopathological conditions, such as wound healing and stromal reaction. According to our data, Raf isoforms and p110γ appear as important mediators of their effects and could therefore qualify as potential therapeutics targets in strategies aiming at limiting elastin contribution to cancer progression. But, because they are expressed in these cells, these signalling molecules are most certainly important modulators of fibroblast physiological functions such as their migration and their proliferation. Thus, the potential for adverse side effects on these stromal cells should be an important consideration when designing such therapies.

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FOOTNOTES

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FIGURES LEGEND

FIG. 1. **Regulation of MEK1/2 and ERK1/2 activation by kE through PKA and PI3K dependent pathways**.

Western-blot analysis of cellular extracts. Membranes were probed with anti-phospho-ERK1/2 (T202/Y204) and anti-phospho-MEK1/2 (S217/S221) polyclonal antibodies. To demonstrate equal loading, blots were stripped and reprobed with an anti-β-actin antibody. Blots are representative of three independent experiments with similar results. *A)* Cells were incubated without (–) or with 50 µg kE/ml (+) for the indicated times. *B)* Cells were stimulated for 30 min. The PI3K (25µM LY294002) and PKA (1µM H-89) inhibitors were added 1h prior to stimulation. EBP antagonist (1 mM lactose) was added 3h before stimulation. The densitometric analysis obtained from the blots is presented. *C)* Cells were stimulated for 30 min. LY303511 (25 µM) and KT5720 (2 µM) were added 1h prior to stimulation.

Data are mean ± SEM, $n = 3$. Significance when compared to the agonist alone : **, $p<0.01$ and ***, $p<0.001$.

FIG. 2. **kE stimulates phosphorylation of S338 Raf-1 and Raf-1 kinase activity in a PI3K-dependent manner.**

A) Fibroblasts were stimulated during the indicated times in absence (–) or presence (+) of kE (50 µg/ml). Membranes were Western-blotted with specific anti-phospho-S338-Raf-1 antibody. To demonstrate equal loading, blots were stripped and reprobed with anti-β-actin. The presented Western-blots are representative of three independent experiments with similar results. *B)* Same as *A* except that LY294002 (25 µM) was preincubated 1 h prior to cell stimulation (30 min). The densitometric analysis is presented under the blot. *C)* Cells were

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stimulated for 30 min with kE (50 µg/ml). LY294002 was used as described above. Equal amounts of proteins were subjected to immunoprecipitation using a Raf-1 specific antibody. Immunoprecipitates were incubated in the presence of GST-MEK1 and [γ-^{32}P]-ATP for 30 min then subjected to SDS-PAGE and autoradiographied. Results are representative of three independent experiments with similar results. The corresponding densitometric analysis is shown.

Data are mean ± SEM, n = 3. Significance when compared to the agonist alone : **, p<0.01 and ***, p<0.001.

FIG. 3. **p110g expression and PI3K activity in dermal fibroblasts.**

A) Untreated fibroblasts lysates were Western-blotted using a p110γ specific antibody. The presented blot is representative of three independent experiments with similar results. *B)* Fibroblasts were treated with kE (50 µg/ml) for 30 min. Equal amounts of proteins were subjected to immunoprecipitation using a p110γ or a p85 specific antibody. Immunoprecipitates were incubated with a mixture of phosphatidylinositol/phosphatidylserine and [γ-^{32}P]-ATP for 15 min. Lipids were extracted, separated using TLC and the plates were autoradiographed. PI-3P was identified by comparing its R_F to that of a commercial control. The presented figure is representative of three independent experiments. The corresponding densitometric analysis is shown. *C)* Fibroblasts were treated with kE (50 µg/ml) for 30 min. LY294002 (25 µM) and LY303511 (25 µM) were added 1h prior to stimulation. Equal amounts of proteins were subjected to immunoprecipitation using a p110γ specific antibody. The PI3K activity assay was performed as described in *B)*.

Data are mean ± SEM, n = 3.***, significantly different (p<0.001) from the corresponding control. NS, not significantly different from the corresponding control.

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FIG. 4. **Effects of PTX on S338-Raf-1 phosphorylation, MEK1/2, ERK1/2 (A) and p110g activation (B). Influence of b ARK1-CT expression on p110g activity (C).**

A) Cells were stimulated for 30 min with kE (50 µg/ml). PTX (100 ng/ml) was preincubated for 18h prior to stimulation. Cellular extracts were Western-blotted using anti-phospho-T202/Y204-ERK1/2, anti-phospho-S217/S221-MEK1/2, and anti-phospho-S338-Raf-1 antibodies. The corresponding densitometric analysis is presented under the blot. Blots are representative of three independent experiments with similar results. *B)* Cell stimulation same as *A*. Cell lysates were subjected to immunoprecipitation using a specific p110γ antibody. Immunoprecipitates were incubated with a mixture of phosphatidylinositol/phosphatidylserine and [γ-^{32}P]-ATP for 15 min. Lipids were extracted, separated using TLC and the plates were autoradiographed. PI-3P was identified by comparing its R_F to that of a commercial control. The presented figure is representative of three independent experiments. The corresponding densitometric analysis is shown. *C)* Fibroblasts were transfected with pRK5-βARK1-CT (βARK1-CT) or with the corresponding empty vector (V), and stimulated for 30 min with kE (50 µg/ml). PI3K activity was determined as in *B*. The presented figure is representative of three independent experiments. The corresponding densitometric analysis is shown.

Data are mean ± SEM, n = 3. Significance when compared to the agonist alone : **, p<0.01 and ***, p<0.001.

FIG. 5. **B-Raf expression and activity in dermal fibroblasts.**

A) Untreated fibroblasts lysates were subjected to Western-blot analysis using a B-Raf antibody. The band observed under the major 95 kDa isoform is ascribed to a spliced variant of B-Raf. The 68 kDa B-Raf isoform was not observed. The blot is representative of three independent experiments with similar results. *B)* Cells were stimulated for 30 min with kE (50 µg/ml). H-89 (1 µM) was preincubated for 1h prior to stimulation. Cell lysates were subjected

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to immunoprecipitation using a B-Raf specific antibody. The immunoprecipitates were incubated with GST-MEK1 and [γ-^{32}P]-ATP for 30 min and further subjected to SDS-PAGE and autoradiographed. The presented results are representative of three independent experiments with similar results. The corresponding densitometric analysis is shown. *C)* Same as *B)* using KT5720 (2 µM). Data are mean ± SEM, n = 3. Significance when compared to the agonist alone : **, p<0.01.

FIG. 6. **ERK activation is independent of Ras.**

A) Cells were cotransfected with constructs encoding HA-tagged-ERK1 and the dominant negative RasN17 or with the corresponding empty vectors (V). After kE stimulation for 30 min (50 µg kE/ml), cells were lysed and HA-tagged-ERK1 was immunoprecipited with an anti-HA antibody. Immunoprecipitates were then subjected to Western-blotting using the anti-phospho-ERK1/2 antibody. The amount of immunoprecipited HA-tagged-ERK1 was controlled by anti-ERK1/2 immunoblotting. Blots are representative of three independent experiments with similar results. The corresponding densitometric analysis is shown. *B)* Same as *A)* except that cells were stimulated with EGF (2 ng/ml) for 5 min.

Data are mean ± SEM, n = 3. NS, not significantly different from the agonist alone.

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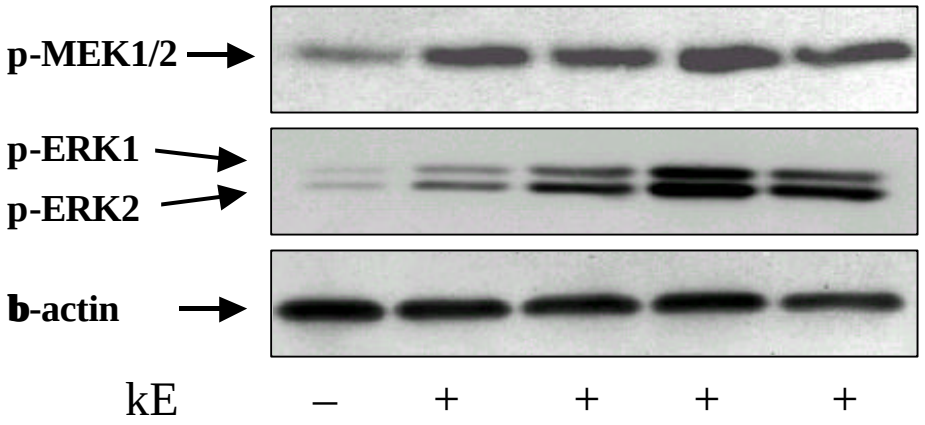

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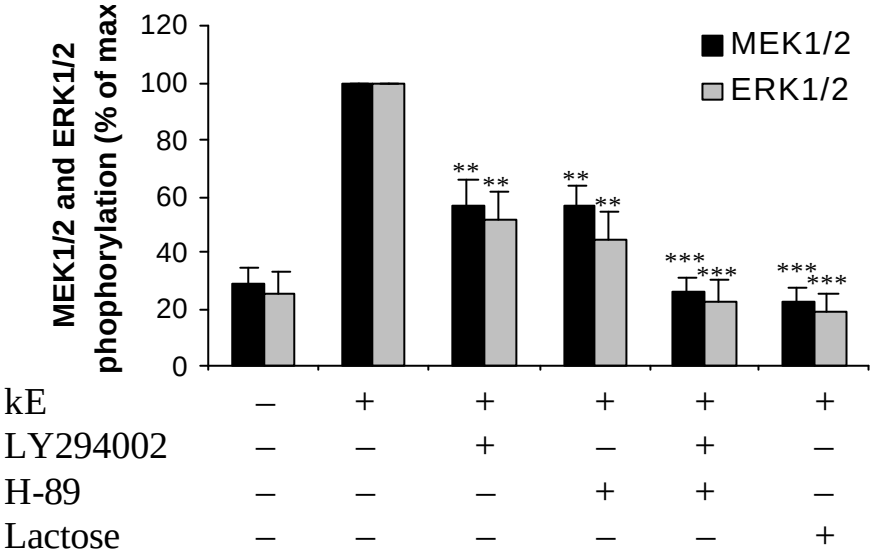

Figures 1A and 1B

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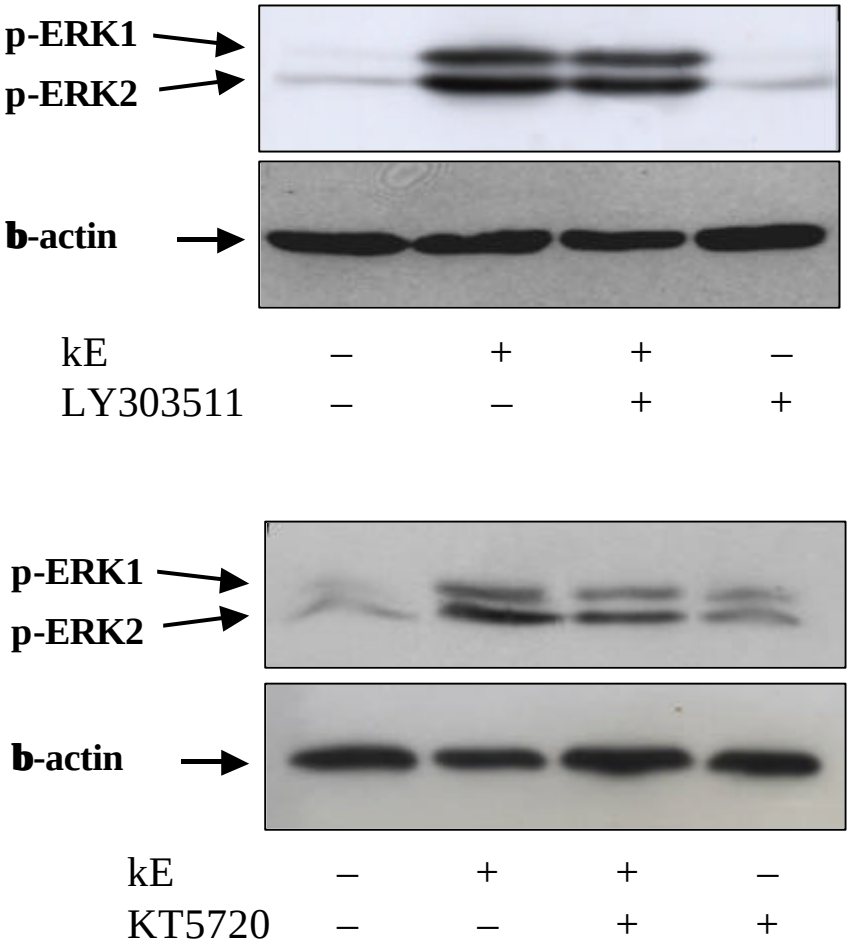

Figure 1C

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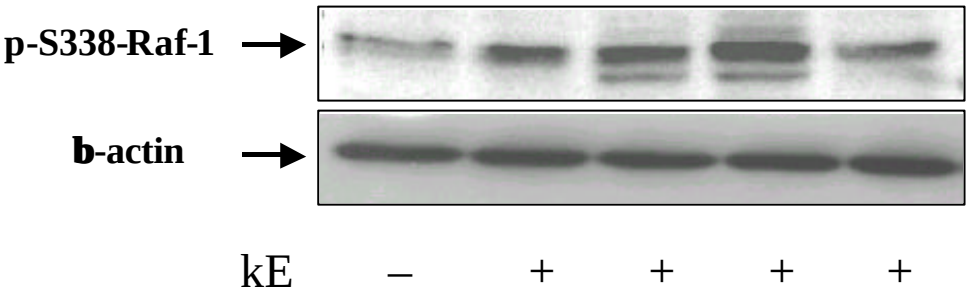

Figure 2A

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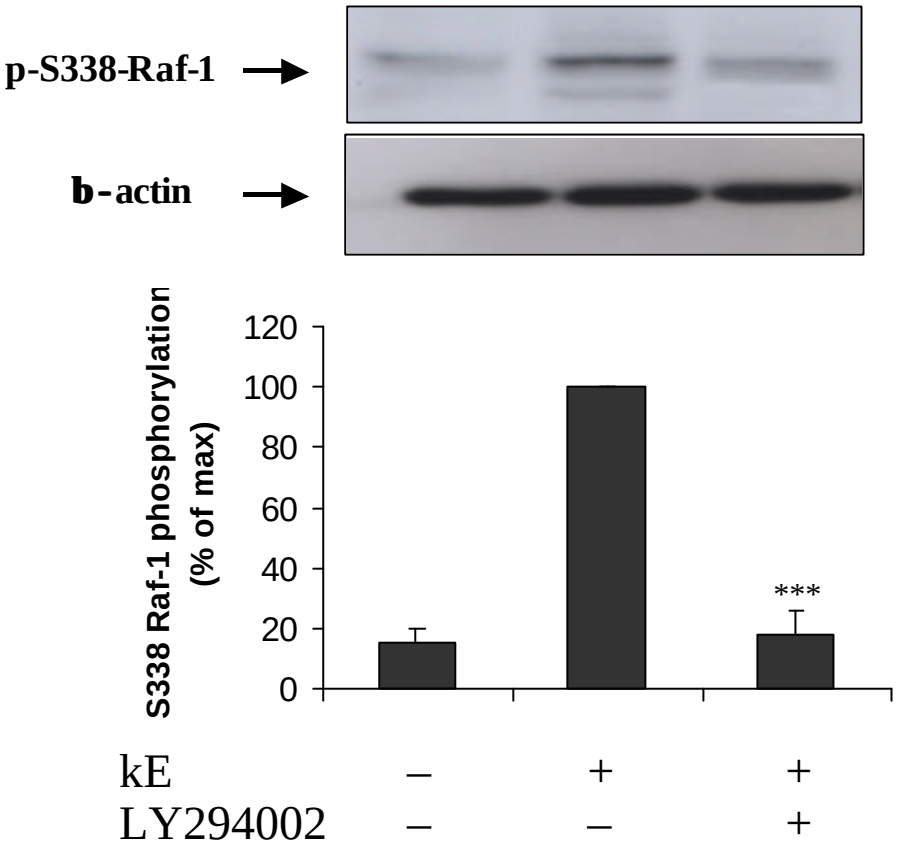

Figure 2B

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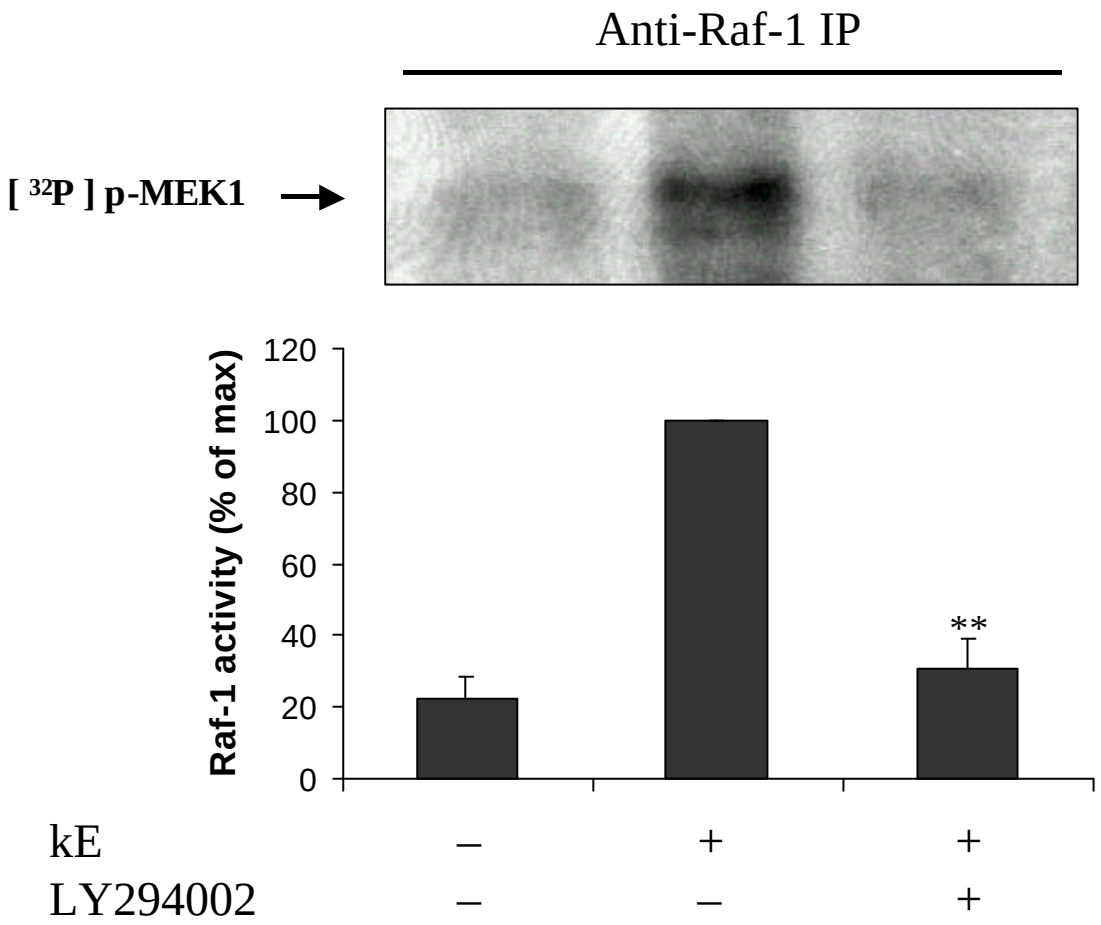

Figure 2C

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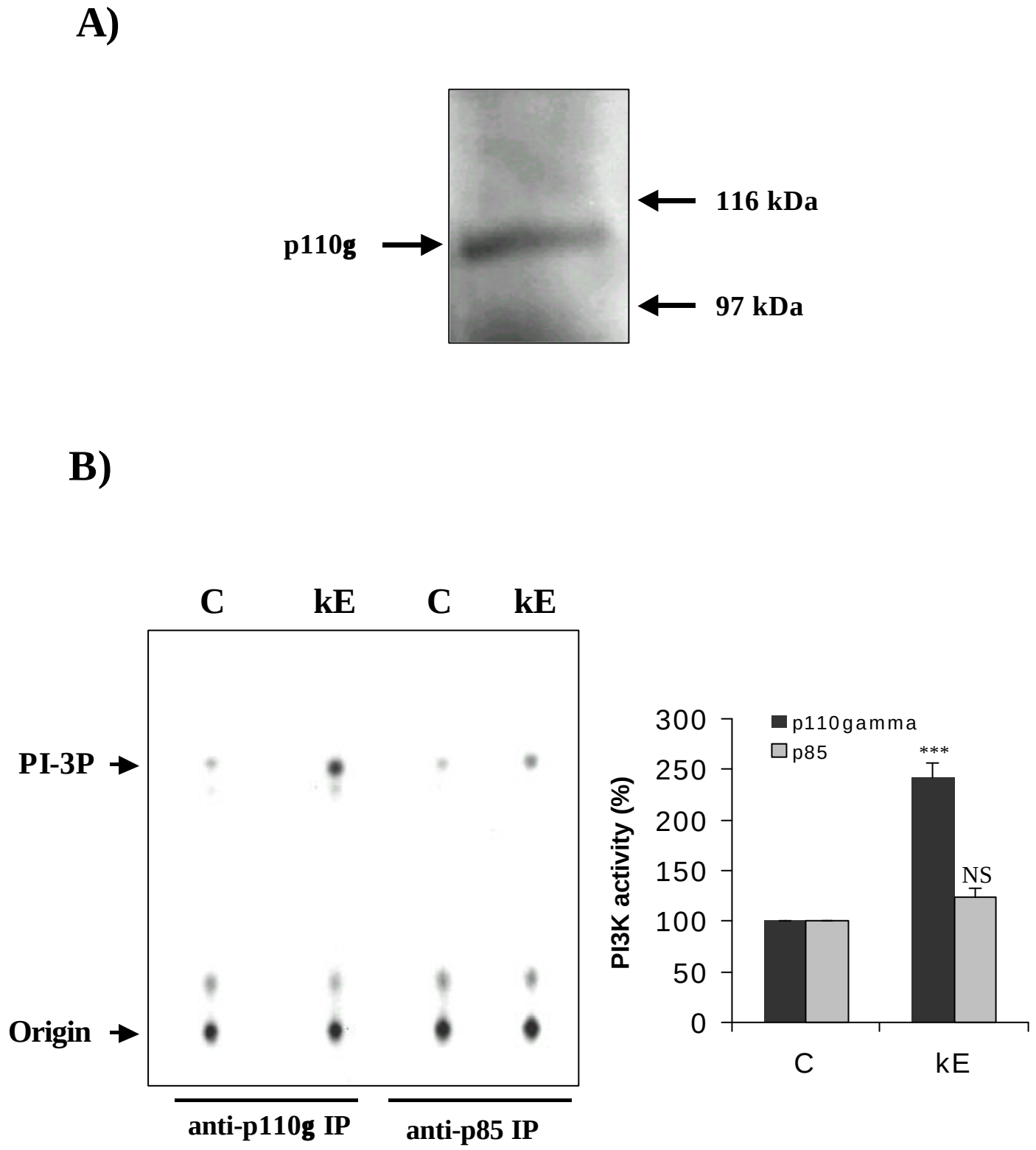

Figures 3A and 3B

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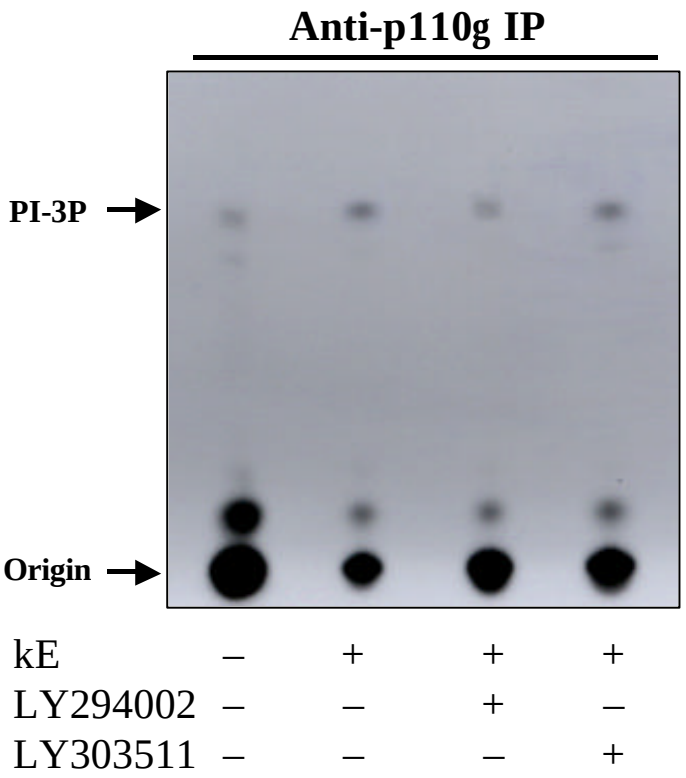

Figure 3C

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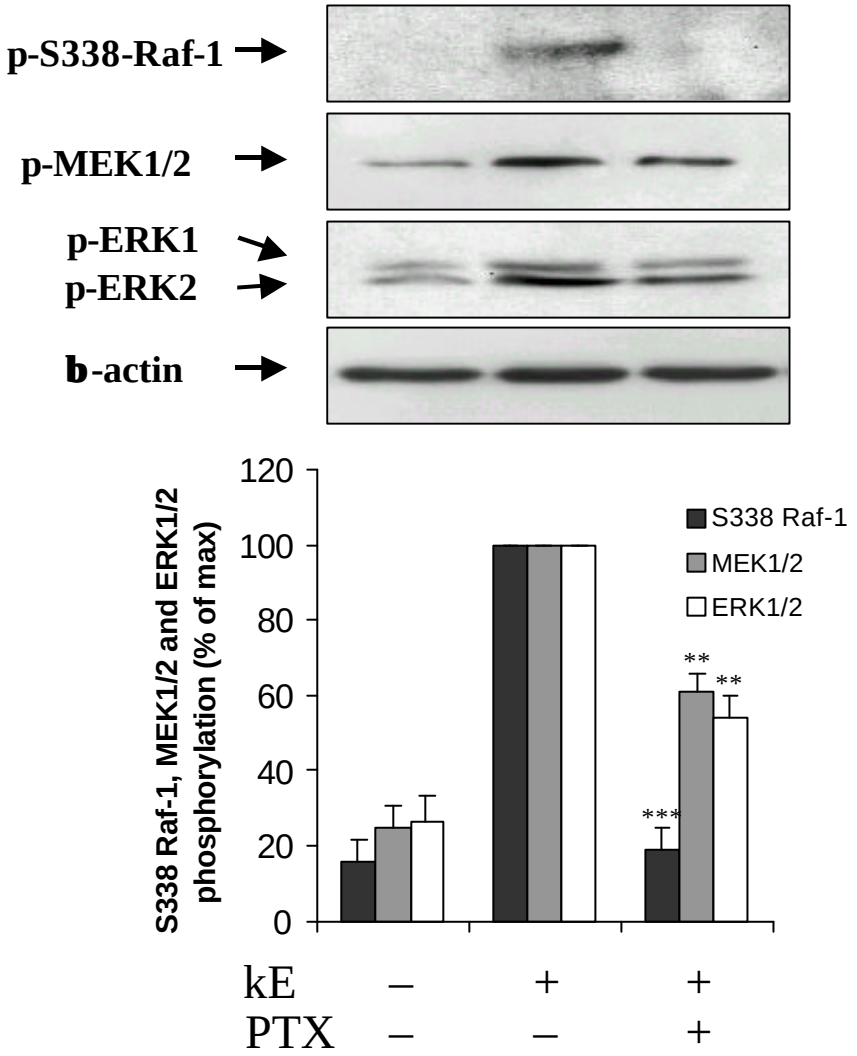

Figure 4A

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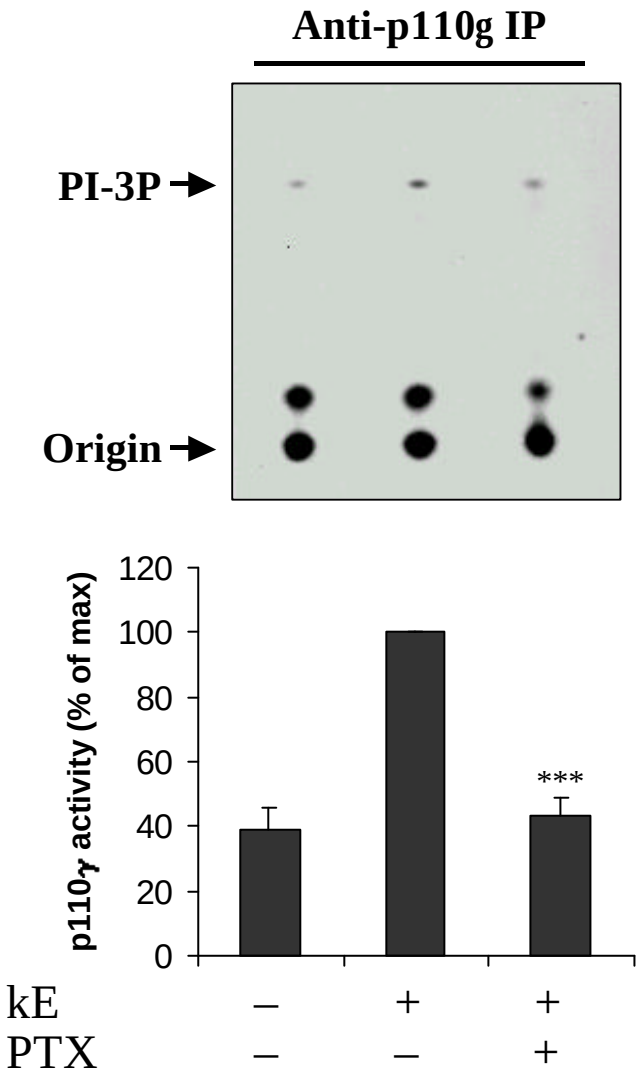

Figure 4B

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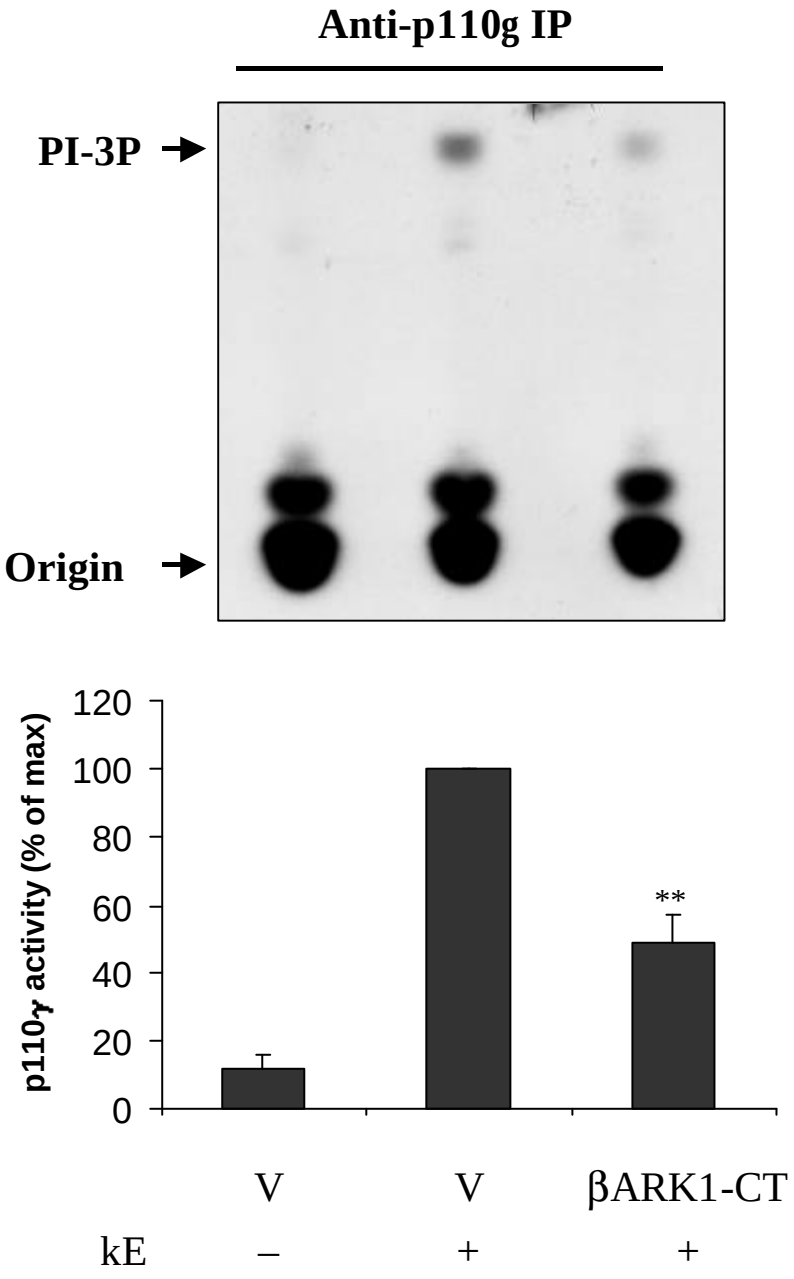

Figure 4C

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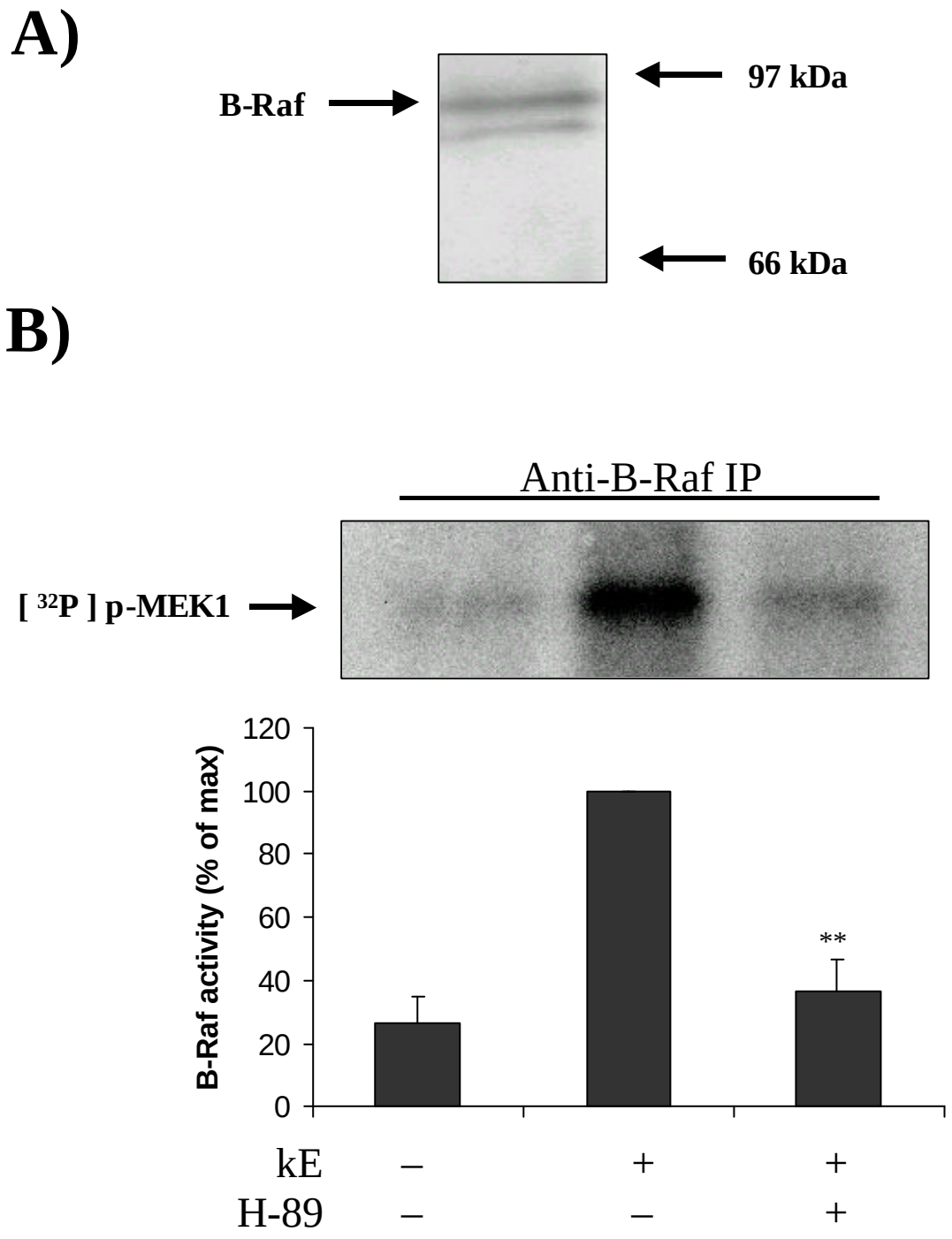

Figures 5A and 5B

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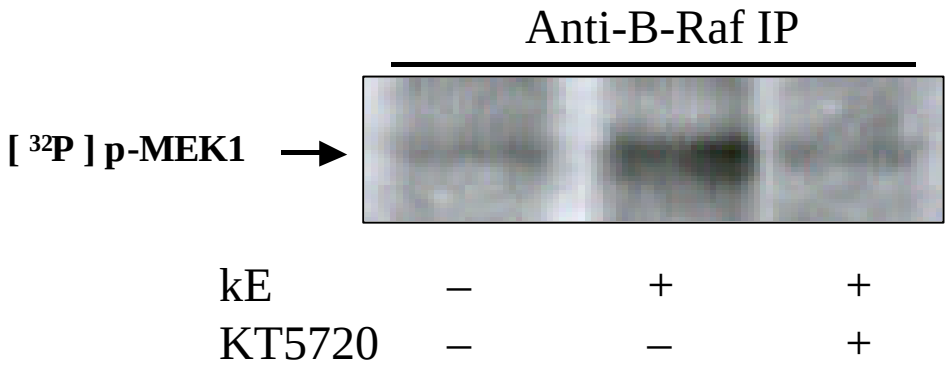

Figure 5C

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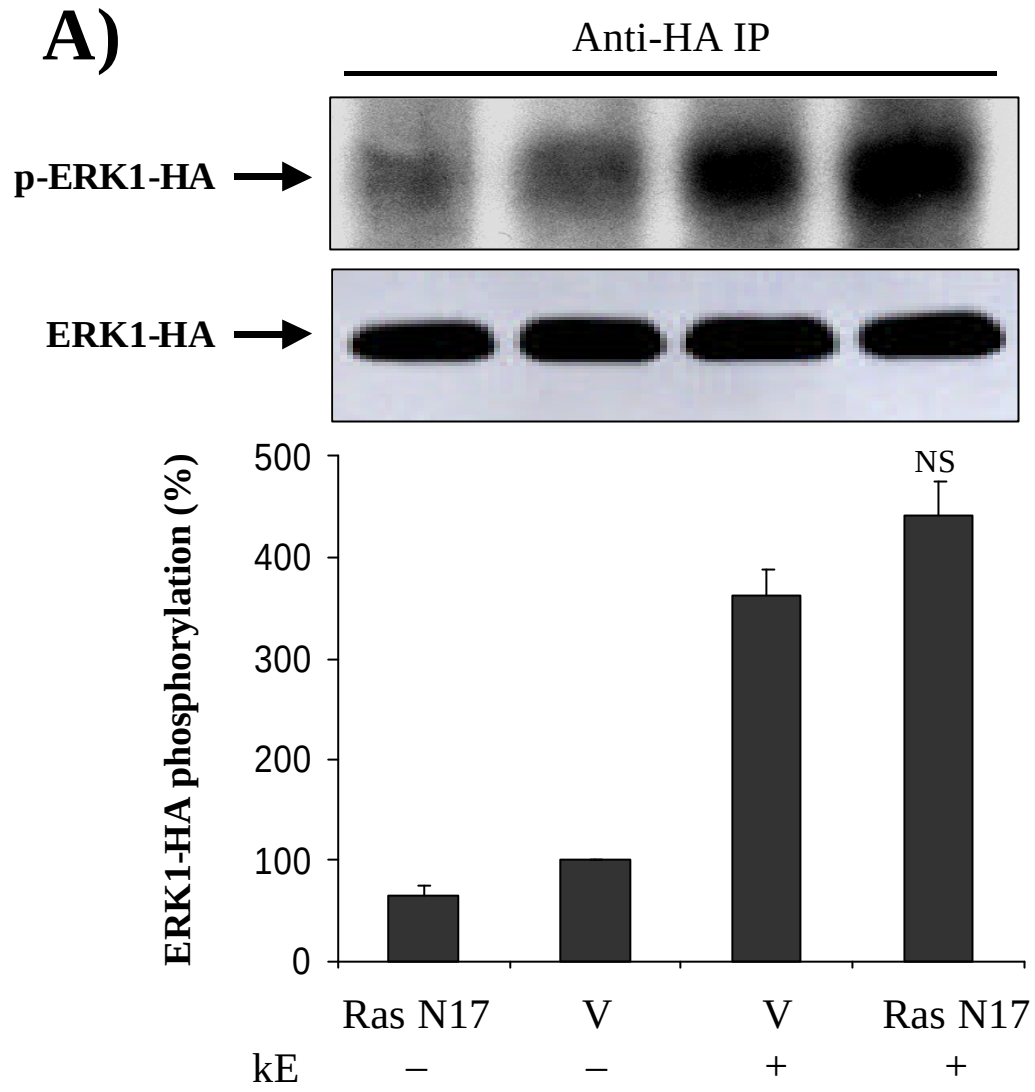

Figure 6

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