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Elastin Peptides Activate Extracellular Signal-Regulated Kinase 1/2 via a Ras-Independent Mechanism Requiring Both p110γ/Raf-1 and Protein Kinase A/B-Raf Signaling in Human Skin Fibroblasts

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ABSTRACT

Elastin peptides (EPs) 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 caused by 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 κ-elastin (κE), a commonly used EP, induces maximum phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)1/2 and ERK1/2 after 30 min. The simultaneous inhibition of PKA and PI3K, by N-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H89) 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. κE 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 after fibroblast stimulation. Pertussis toxin also blocks the Raf-1/MEK1/2/ERK1/2 phosphorylation cascade. In addition, we found that B-Raf is expressed in dermal fibroblasts and activated in a PKA-dependent manner after κE treatment, thereby integrating PKA signals to MEK1/2. It is noteworthy that Ras involvement was excluded because ERK1/2 activation by κE was not blocked in RasN17-transfected fibroblasts. Together, 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 seem to be important modulators of dermal fibroblasts physiology and should now qualify as therapeutic targets in strategies aiming at limiting elastin degradation contribution to cancer progression.

Elastin is the extracellular matrix molecule responsible for resilience of tissues and was first 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 (EPs), is a peripheral 67-kDa pro-

ABBREVIATIONS: EP, elastin peptide; EBP, elastin binding protein; κE, κ-elastin; ERK, extracellular-signal regulated kinase; PKA, protein kinase A; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; EGF, epidermal growth factor; PTX, pertussis toxin; H89, N-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide; LY294002, 2-(4-morpholynil)-8-phenyl-4H-1-bemzo-pyran-4-one; LY303511, 2-piperazinyl-8-phenyl-4H-1-benzopyran-4-one; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; βARK1-CT, C-terminal domain of the β-adrenergic receptor kinase 1; PBS, phosphate-buffered saline; TLC, thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; SMC, smooth muscle cell; KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester.

tein, 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 (κE), up-regulates the expression of procollagenase-1, whose 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 phosphorylation by dual-specific kinases named mitogen-activated protein kinase/extracellular signal-regulated kinases 1 and 2 (MEK1/2), which are 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, in which expressions are more restricted. Compared with Raf-1, B-Raf is a stronger activator of the ERK pathway, whereas 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 or PI3K have also been reported (Dhillon and Kolch, 2002). The phosphorylation of Raf-1 on Ser338 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; Takeda et al., 1999; Mas et al., 2003).

Class I$_A$ PI3Ks 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$_B$ PI3Ks possess a p101 regulatory subunit associated to a p110γ catalytic subunit, which can be directly activated by G protein $\beta\gamma$ subunits or Ras (Anderson and Jackson, 2003). Class I$_A$ PI3Ks 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, whereas 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 occur via two mechanisms involving either the cAMP receptor 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

NIH 3T3 fibroblasts (Vossler et al., 1997; Huser et al., 2001), 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 κE 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. In addition, we show that p110γ and B-Raf, which had never been reported in dermal fibroblasts, are important modulators of the physiology of these cells. Because their involvement could explain the strong and sustained activation of ERK1/2 and subsequent procollagenase-1 production, their control in strategies aiming at limiting elastin peptides contribution to cancer progression is discussed.

Materials and Methods

Materials. κE was prepared as described previously (Brassart et al., 2001). Human recombinant epidermal growth factor (EGF) was from Upstate Biotechnology (Lake Placid, NY) (distributed by Euromedex, Mundelsheim, France). Lactose, pertussis toxin (PTX), phosphatidylserine, phosphatidylinositol, phosphatidylinositol 3-phosphate, and proteases inhibitors cocktail (reference P8340) were from Sigma (Saint-Quentin Fallavier, France). H89, KT5720, and LY303511 were from Calbiochem (distributed by VWR International, Strasbourg, France). LY294002 was purchased from Cell Signaling Technology Inc. (distributed by Ozyme, Saint-Quentin en Yvelines, France). Recombinant GST-MEK1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (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 Thr202 and Tyr204), MEK1/2 (phosphorylated on Ser217 and Ser221), and phospho-specific antibody against phosphorylated Raf-1 Ser338 and anti-ERK1/2 antibody were from Cell Signaling Technology Inc. (Beverly, MA). Rabbit anti-p110γ, anti-Raf-1, and anti-B-Raf polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Polyclonal anti-p85 antibody was from Upstate Biotechnology (distributed by Euromedex). Mouse monoclonal anti-hemagglutinin (HA) tag was from Roche Diagnostics (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 Inc. (Orsay, France). Others reagents were from Sigma.

Expression Plasmids. The plasmid pRK5-βARK1-CT (Koch et al., 1994), which encodes the Gly495-Leu689 fragment corresponding to the C terminus of β-adrenergic receptor kinase 1 (βARK1-CT), was kindly provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC). The constructs encoding HA-tagged-ERK1 (pECE-HA-ERK1) and the dominant negative RasN17 mutant (pSV-RasN17) were described previously (Guillemot et al., 2000) and were kind gifts from Dr. J. Pouysségur (Institute of Signaling, Developmental Biology and Cancer Research, Nice, France) and Dr. F. Schweighoffer (ExonHit Therapeutics, S.A., Paris, 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 (aged 21–49 years). Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine in the presence of 5% CO_2. Cells at subcultures 5 to 10 were used. For experiments, fibroblasts were grown to subconfluence 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, and then incubated in serum-free DMEM with or without κE (50 μg/ml) or EGF (2 ng/ml)

for the indicated times. The pharmacological inhibitors H89 (1 μM), KT5720 (2 μM), LY294002 (25 μM), and LY303511 (25 μM) were preincubated 1 h before stimulation, whereas lactose (1 mM) and PTX (100 ng/ml) were preincubated 3 and 18 h, respectively. Elastin peptide stimulation was stopped by adding ice-cold PBS containing 50 μM Na$_3$VO$_4$. For transfection experiments, cells were grown to 80 to 90% of confluence 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 of 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.

Western Blotting. Cells (10^6) were washed twice in ice-cold PBS containing 50 μM Na$_3$VO$_4$, 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$_3$VO$_4$, and protease inhibitor cocktail). Insoluble material was removed by centrifugation (20,000g, 20 min, 4°C). Protein concentrations were determined by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL; 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) nonfat dry milk in Tris-buffered saline/Tween 20 (50 mM Tris, pH 7.5, 150 mM NaCl, and 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-Ser338-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 Tris-buffered saline/Tween 20, the membranes were incubated for 1 h at room temperature in the presence of horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (1:4000 and 1:10,000 in blocking buffer, respectively). Immunocomplexes were detected by chemiluminescence. Blots were semiquantified 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$_3$VO$_4$, scrapped in this buffer, and centrifuged (375g, 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, 1 mM Na$_3$VO$_4$, and proteases inhibitor cocktail]. Insoluble material was removed by centrifugation (20,000g, 20 min, 4°C). Protein concentrations were determined using bicinchoninic acid protein assay, and equal amounts of proteins were incubated with 2.5 μg of anti-p85 or anti-p110γ antibodies for 1 h at 4°C. The antigen-antibody complexes were incubated with protein G Plus-Sepharose (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer, and then twice with lipid kinase buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$, and 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 of [γ-^{32}P]ATP. The reaction was performed for 15 min at 30°C and stopped by adding 100 μl of 1 M 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/1 M 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$_2$O [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 κE 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 1 h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer, and then twice with kinase buffer (20 mM HEPES, pH 7.2, 10 mM MgCl$_2$, and 10 mM MnCl$_2$). For kinases activity assays, pellets were resuspended in 50 μl of kinase buffer supplemented with 1 μg of GST-MEK1, 2.5 μM ATP, and 10 μCi of [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.

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 before stimulation. Equal amounts of proteins were incubated for 1 h at 4°C with 3 μg of anti-HA tag antibody. The formed antigen-antibody complexes were incubated with protein G Plus-Sepharose for 1 h at 4°C, collected by centrifugation, washed three times in immunoprecipitation lysis buffer, and then resuspended in Laemmli sample buffer. Protein 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 $\pm$ S.E.M. Comparison between groups were made using Student's t test. The results were considered significantly different at $p < 0.05$.

Results

κE Induces MEK1/2 Activation via PI3K and PKA-Dependent Signaling. In previous work, we have shown that the treatment of human skin fibroblasts with κE 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 κE, 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 by using an antibody specifically recognizing its active form (i.e., phospho-Ser217/Ser221-MEK1/2). In parallel, the presence of the phospho-Thr202/Tyr204-ERK1/2 active form was checked.

The treatment of fibroblasts with 50 μg of κE/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). It is noteworthy that a strong activation of both kinases still persists after 60 min of stimulation.

The importance of PKA and PI3K signaling toward MEK1/2 activation was investigated using pharmacological inhibitors. When cells were pretreated for 1 h with H89 (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 abolished MEK1/2 and ERK1/2 activation (Fig. 1B). The same effect was obtained using 1 mM lactose (Fig. 1B).

To support these observations, κE-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 H89. Our results show (Fig. 1C) that LY303511 has no effect on κE-induced ERK1/2 activation, whereas the use of KT5720 reproduced the effect of H89, supporting our previous observations. PKA and PI3K thus seemed to be crucial modulators of the MEK1/2/ERK1/2 cascade in elastin peptide-induced signaling.
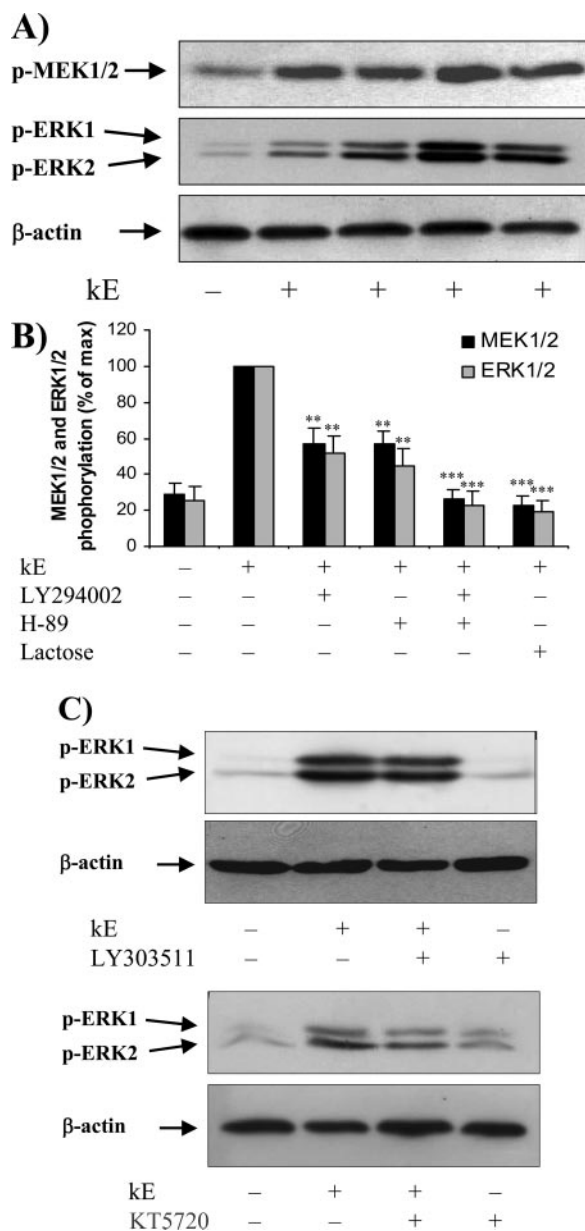

Fig. 1. Regulation of MEK1/2 and ERK1/2 activation by κE through PKA- and PI3K-dependent pathways. Western blot analysis of cellular extracts. Membranes were probed with anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-phospho-MEK1/2 (Ser217/Ser221) polyclonal antibodies. To demonstrate equal loading, blots 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 of κE/ml (+) 5, 15, 30, and 60 mins. B, cells were stimulated for 30 min. The PI3K (25 μM LY294002) and PKA (1 μM H89) inhibitors were added 1 h before stimulation. EBP antagonist (1 mM lactose) was added 3 h 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 1 h before stimulation. Data are mean ± S.E.M., $n = 3$. Significance compared with the agonist alone: ∗∗, $p < 0.01$ and ∗∗∗, $p < 0.001$.

Inhibition of PI3K Blocks κE-Induced Raf-1 Ser338 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 Ser338 (Mason et al., 1999). Several authors have suggested that Ser338 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 κE on Ser338 phosphorylation and on Raf-1 activity in presence of LY294002. Using an anti-phospho-Ser338-Raf-1 antibody, we show by Western blot that κE cell stimulation leads to Ser338 Raf-1 phosphorylation (Fig. 2A) with a time course very similar to that observed for MEK1/2 and ERK1/2 (Fig. 1A). The maximum phosphorylation level was observed at 30 min. The inhibition of PI3K with LY294002 (25 μM) totally blocked Ser338 phosphorylation induced by κE treatment (Fig. 2B). However, even if phosphorylation of Ser338 is required for Raf-1 activation, it is not a surrogate marker for Raf-1 activity (Mason et al., 1999; Chiloeches et al., 2001). 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 were treated with κE, its activity was raised 5-fold (Fig. 2C, second lane). Pretreatment of cells with LY294002 totally blocked Raf-1 activity. PKA inhibition (1 μM H89) had no effect on Raf-1 activity (data not shown).

These results indicated that κE induced Raf-1 Ser338 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.

κE 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; Takeda et al., 1999; Mas et al., 2003). PI3K activity of these two subfamilies was therefore analyzed after κE 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 hematopoietic cells, but its presence has also been reported in nonhematopoietic 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 κE treatment led to an important augmentation of PI3K activity in p110γ immunoprecipitates (approximately 150% increase compared with control). A nonsignificant increase was observed in p85 immunoprecipitates.

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. In addition, Fig. 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. 1, B and C).

p110γ Is Activated by βγ 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). Because the ERK1/2 pathway and p110γ activation can be caused by such a G protein (Takeda et al., 1999; Yart et al., 2002), we analyzed the effects of PTX treatment on ERK1/2 pathway and p110γ activation after elastin peptides stimulation.

Pretreatment with PTX (100 ng/ml, 18 h) totally blocked Raf-1 Ser338 phosphorylation, whereas MEK1/2 Ser217/221

and ERK1/2 Thr202/Tyr204 phosphorylations were partially inhibited (Fig. 4A). Because these observations were similar to those obtained with PI3K blocking (Figs. 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, 18 h) returned κE-induced p110γ activity to control level (Fig. 4B). These data show that a PTX-sensitive G protein is required for p110γ activation.

At first, 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
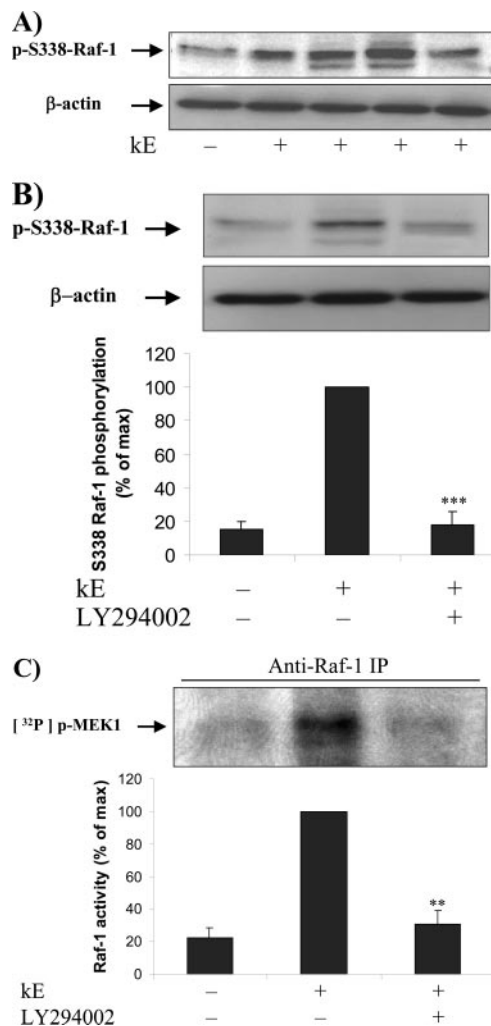

Fig. 2. κE stimulates phosphorylation of Ser338 Raf-1 and Raf-1 kinase activity in a PI3K-dependent manner. A, fibroblasts were stimulated in absence (−) or presence (+) of κE (50 μg/ml) for 5, 15, 30, and 60 min. Membranes were Western blotted with specific anti-phospho-Ser338-Raf-1 antibody. To demonstrate 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 before cell stimulation (30 min). The densitometric analysis is presented under the blot. C, cells were stimulated for 30 min with κE (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-32P]ATP for 30 min and then subjected to SDS-PAGE and autoradiographed. Results are representative of three independent experiments with similar results. The corresponding densitometric analysis is shown. Data are mean ± S.E.M., n = 3. Significance compared with the agonist alone: ∗∗, p < 0.01 and ∗∗∗, p < 0.001.
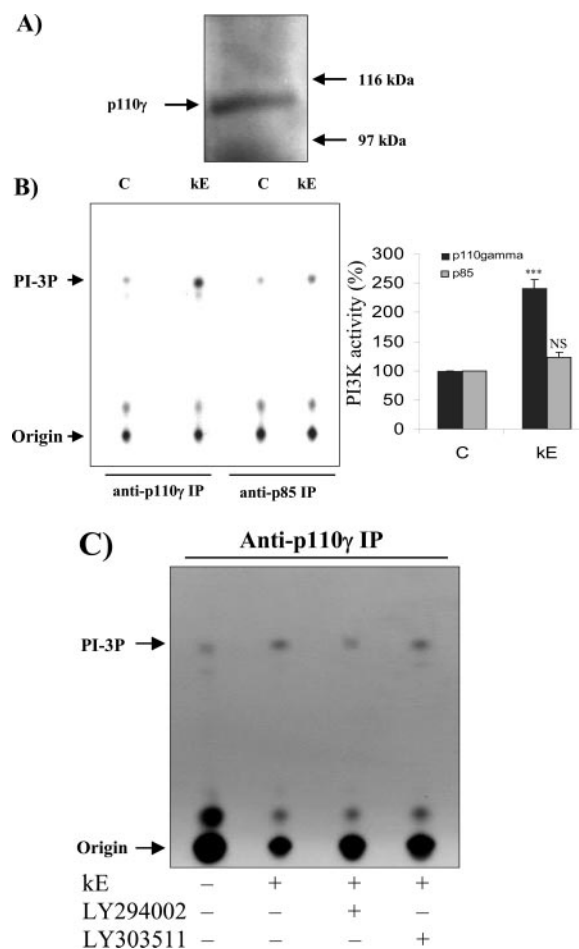
Fig. 3. p110γ expression and PI3K activity in dermal fibroblasts. A, untreated fibroblasts lysates were Western-blotted using a p110γ-specific antibody. The presented blot is representative of three independent experiments with similar results. B, fibroblasts were treated with κE (50 μg/ml) for 30 min. 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-32P]ATP for 15 min. Lipids were extracted, separated using TLC, and the plates were autoradiographed. PI-3-phosphate 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 κE (50 μg/ml) for 30 min. LY294002 (25 μM) and LY303511 (25 μM) were added 1 h before 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 ± S.E.M., n = 3. ∗∗∗, significantly different (p < 0.001) from the corresponding control. N.S., not significantly different from the corresponding control.

(Brock et al., 2003). So, we investigated the possible role of $\beta\gamma$ subunits in p110γ activation.

To test this hypothesis, human skin fibroblasts were transiently transfected with a plasmid encoding the βARK1-CT, which acts as a $\beta\gamma$-scavenger molecule (Koch et al., 1994). The expression of the βARK1-CT polypeptide significantly inhibited κE-induced p110γ activation (Fig. 4C), showing that $\beta\gamma$ subunits from PTX-sensitive G proteins are required for p110γ activation. It is important to underline here that the inhibition level reported in Fig. 4C only reflects the inhibition of this kinase in pRK5-βARK1-CT-transfected fibroblasts. Together, our data show that the treatment of human skin fibroblasts with κE triggers a PTX-sensitive G protein whose $\beta\gamma$ 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 with Raf-1 (Mercer and Pritchard, 2003), but, although its presence was reported in mouse embryonic fibroblasts (Huser et al., 2001), it is absent in NIH 3T3 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.
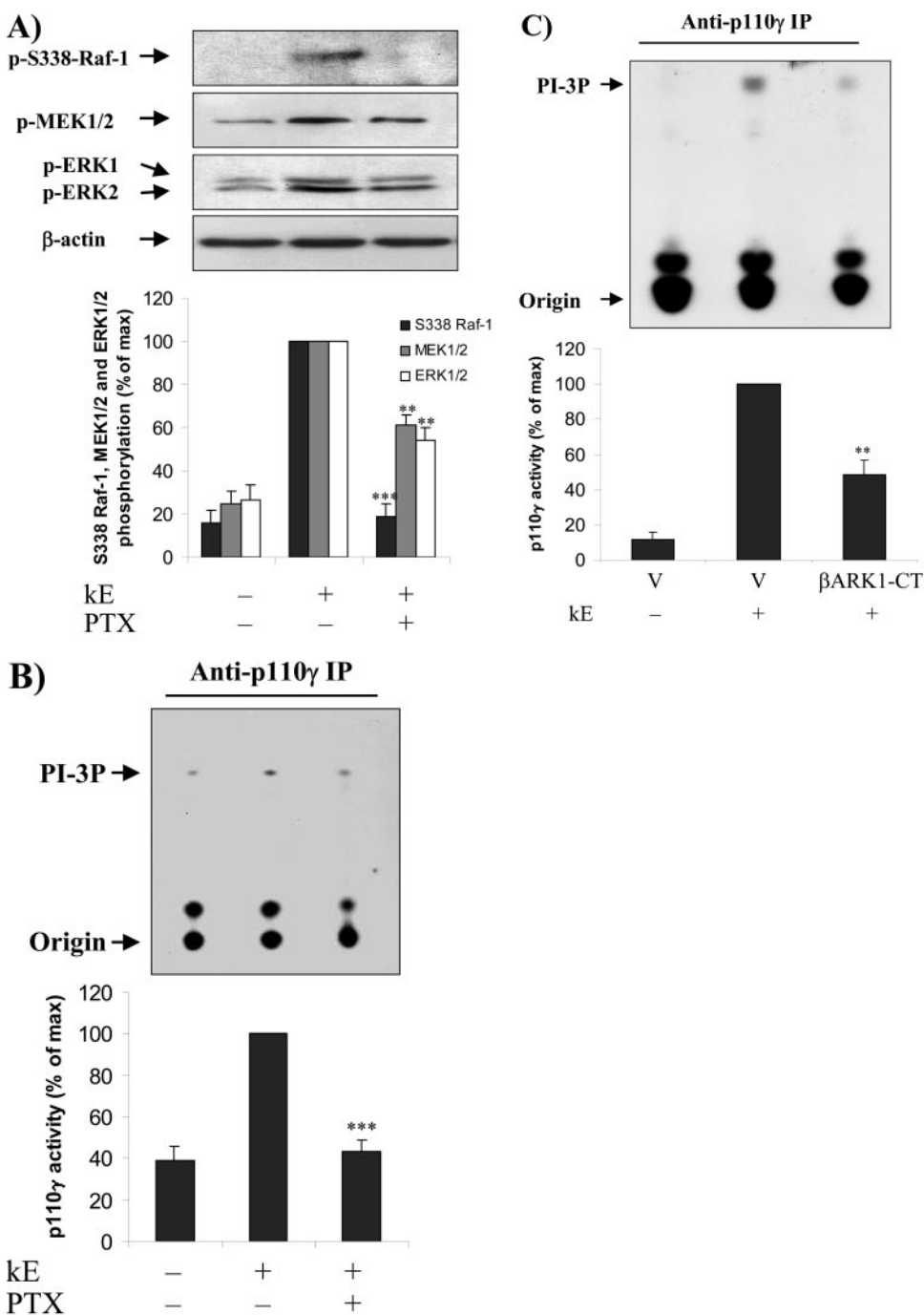

Fig. 4. Effects of PTX on Ser338-Raf-1 phosphorylation, MEK1/2, ERK1/2 (A) and p110γ activation (B). Influence of βARK1-CT expression on p110γ activity (C). A, cells were stimulated for 30 min with κE (50 μg/ml). PTX (100 ng/ml) was preincubated for 18 h before stimulation. Cellular extracts were Western blotted using anti-phospho-Thr202/Tyr204-ERK1/2, anti-phospho-Ser217/Ser221-MEK1/2, and anti-phospho-Ser338-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-3-phosphate 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 κE (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 $\pm$ S.E.M., $n = 3$. Significance compared with the agonist alone: **, $p < 0.01$ and ***, $p < 0.001$.

Because 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 H89.

In resting cells, B-Raf activity was weak (Fig. 5B, lane 1) but strongly increased (5-fold) after κE treatment (Fig. 5B, lane 2). The addition of H89 (1 μM, pretreatment for 1 h) 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.

κE-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 (Suire et al., 2002; Chong et al., 2003; Mercer and Pritchard, 2003).

To evaluate the contribution of Ras in κE-induced ERK1/2 activation, skin fibroblasts were transiently cotransfected with HA-tagged-ERK1 and a dominant-negative Ras, RasN17, and further stimulated with κE 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 overexpression of RasN17 did not block ERK1 phosphorylation in κE-stimulated cells (Fig. 6A, lane 4). A nonsignificant increase in ERK1 phosphorylation was observed when RasN17-transfected cells were stimulated with κE. 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 with lane 3). These results show that the activation of the ERK pathway observed after treatment of human skin fibroblasts with κE is independent of Ras.
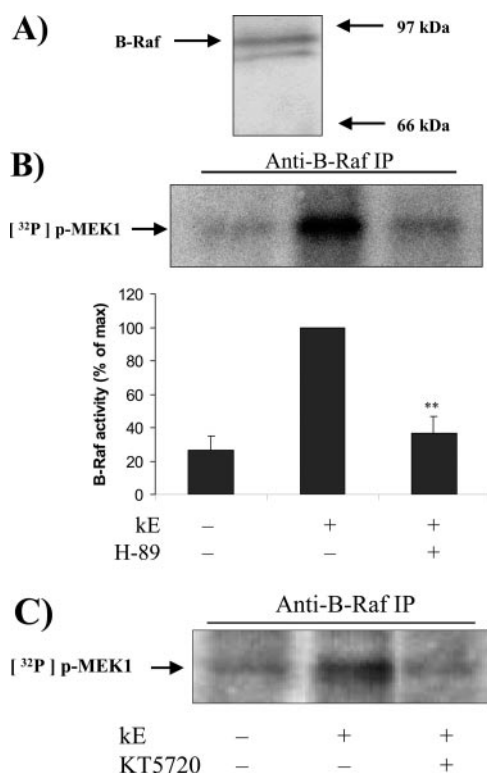

Fig. 5. B-Raf expression and activity in dermal fibroblasts. A, untreated fibroblasts lysates were subjected to Western blot analysis using a B-Raf antibody. The band observed under the major 95-kDa isoform is ascribed to a spliced variant of B-Raf. The 68-kDa B-Raf isoform was not observed. The blot is representative of three independent experiments with similar results. B, cells were stimulated for 30 min with κE (50 μg/ml). H89 (1 μM) was preincubated for 1 h before stimulation. Cell lysates were subjected 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 $\pm$ S.E.M., $n = 3$. Significance compared with the agonist alone: **, $p < 0.01$.
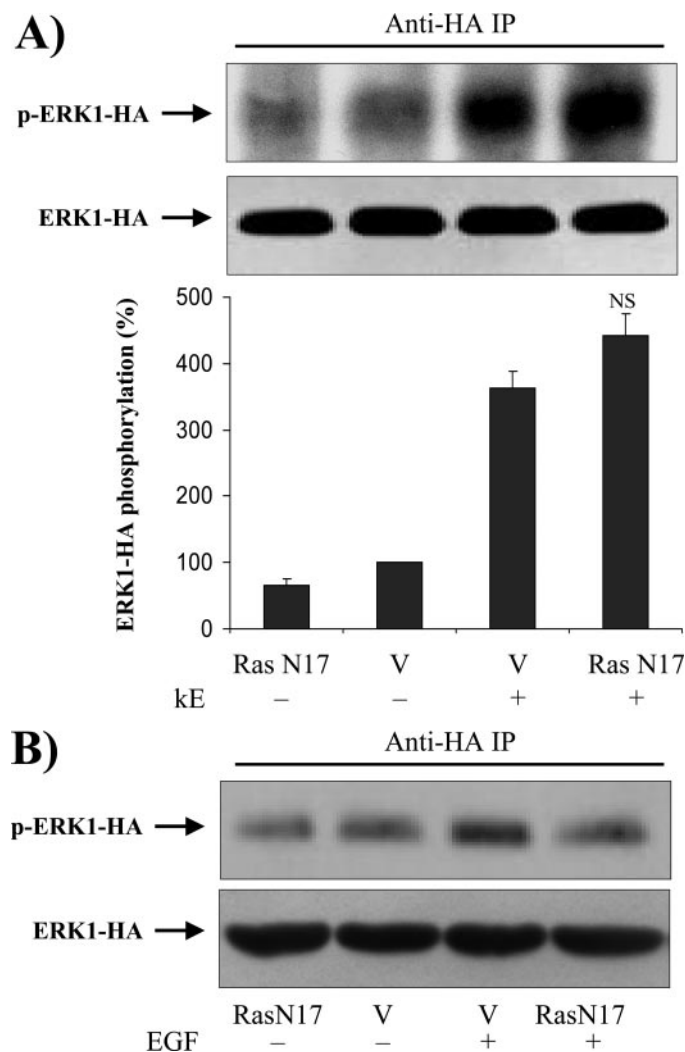

Fig. 6. ERK activation is independent of Ras. A, cells were cotransfected with constructs encoding HA-tagged-ERK1 and the dominant negative RasN17 or with the corresponding empty vectors (V). After κE stimulation for 30 min (50 μg of κE/ml), cells were lysed and HA-tagged-ERK1 was immunoprecipitated with an anti-HA antibody. Immunoprecipitates were then subjected to Western blotting using the anti-phospho-ERK1/2 antibody. The amount of immunoprecipitated 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 $\pm$ S.E.M., $n = 3$. N.S., not significantly different from the agonist alone.

Discussion

The in vivo generation of EPs 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 procollagenase-1 (Brassart et al., 2001), their contribution to metastasis development could be accentuated in such conditions.

In skin fibroblasts, κE up-regulates 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 κE 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 EPs has been reported in smooth muscle cells (SMCs) (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 κE treatment leads to the phosphorylation of MEK1/2 on Ser217/221, 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). Because 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 Ser338 and its kinase activity are controlled by PI3K activity and lead to MEK1/2 activation (Fig. 2).

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

It is well established that phosphorylation of Raf-1 on Ser338 is essential for its activation (Mason et al., 1999). The contribution of PI3K to these events has been proposed previously (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 Ser338 phosphorylation only at concentrations far greater than those required to inhibit PI3K. They thus suggested that Raf-1 Ser338 phosphorylation inhibition could not be attributed to PI3K.

In this study, we blocked Ser338 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, Ser338 phosphorylation could be PI3K-dependent, which supports the hypothesis that PI3K could contribute to Raf-1 Ser338 phosphorylation and activation (Chaudhary et al., 2000; Sun et al., 2000).

We analyzed the effects of κE stimulation on the activities of class I PI3K. In dermal fibroblasts, the activity of class I_A isoforms remained unchanged after κE 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 Ser338, MEK1/2, and ERK1/2 phosphorylation with an efficiency comparable with 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 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 preactivation 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 nonsignificant. So, the participation of p110β to ERK1/2 activation was excluded. In addition, Raf-1 Ser338 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).

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

We have shown that κE 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 kinase activity on MEK was strongly increased by κE and could be totally blocked using H89 or KT5720 (Fig. 5, B and C). Thus, B-Raf activation seemed to be 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 also should be explored. Indeed, PTX-sensitive G protein βγ subunits can activate the adenylyl cyclase (Albert and Robillard, 2002). However, such a mechanism cannot 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_o in EP signaling (Brassart et al., 2001; Mochizuki et al., 2002). Our work supports 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 attributable to our ignorance of its operational mechanism.

It has been suggested that EP could lead to Ras induction in porcine SMCs (Mochizuki et al., 2002). Moreover, Ras can directly activate Raf-1, B-Raf, and p110γ (Suire et al., 2002; Mercer and Pritchard, 2003). 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 κE-induced ERK activation does not require Ras (Fig. 6A). The slight increase in HA-tagged-ERK-1 phosphorylation we observed when κE-treated fibroblasts were transfected with RasN17 was nonsignificant. A comparable, but more important effect, was reported for nocodazole-treated RasN17-transfected human embryonic kidney 293T 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 κE-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 κE. EPs 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.

EPs are produced in normal and physiopathological conditions, such as wound healing and stromal reaction. According to our data, Raf isoforms and p110γ seem to be 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 signaling 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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