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Protein Phosphatase 2A Regulatory Subunit Bβ Promotes MAP Kinase-mediated Migration of A431 Cells

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Running head: PP2A/Bβ promotes MAP kinase-mediated migration

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Abstract

Background/Aims: Phosphatases are involved in regulation of MAP kinase (MAPK). A431 cells migrate on collagen after EGF stimulation using MAPK. To clarify the involvement of PP2A in this MAPK-dependent migration, the expression of an isoform of the B regulatory subunit was inhibited. *Methods:* An antisense sequence corresponding to Bβ cDNA was transfected into A431 cells. Their migratory activity on collagen was examined using Transwell, and MAPK phosphorylation and phosphatase activity were measured, and the results were compared with those obtained with mock-transfected cells. *Results:* Antisense-transfected cells showed less Bβ protein and phosphatase activity than mock-transfected controls. Migration of antisense-transfected cells showed a low response to EGF. The response of MAPK phosphorylation of antisense-transfected cells to EGF stimulation and adhesion to collagen in the presence or absence of EGF were markedly decreased. Phosphatase activity of PP2A-Bβ also did not respond to EGF, collagen or EGF plus collagen, and remained at low levels. *Conclusion:* These results suggested that PP2A-Bβ promotes cell migration through the MAPK cascade.

Introduction

Cell migration requires stimulation by a motogenic cytokine and cell adhesion to the extracellular matrix, which is mediated by integrins [1-3]. Epidermal growth factor (EGF) is a motogenic cytokine for a variety of cell types [4, 5]. EGF-induced signalling for migration begins with binding of EGF to EGF receptors, which then deliver a signal to a protein kinase cascade involving Raf-1 kinase, MEK and MAP kinase (MAPK) *via* Ras [6, 7]. Cell adhesion to the extracellular matrix induces assembly of distinct integrins at focal contacts and also activates the MAPK cascade [8, 9]. EGF and adhesion to collagen (COL) activate MAPK synergistically in A431 cells, and the resultant sustained MAPK activation leads to migration [10].

The activation of kinases is controlled by phosphatases [11-13]. A balance of kinases and phosphatases is necessary for cells to maintain an appropriate level of the transducing signal for migration. However, phosphatases have been studied less intensively than kinases, and the regulatory mechanism of MAPK activation by phosphatases is not yet fully understood. Both serine/threonine phosphatases and a serine/threonine/tyrosine phosphatase are likely to be involved in regulation of the MAPK cascade [14-19]. On the other hand, the serine/threonine phosphatases PP1 and PP2A are known to regulate cell motility [20-22], and several studies have indicated that PP2A regulates MAPK signalling [15, 16, 23].

PP2A is a holoenzyme consisting of a heterotrimer of a 65-kDa structural subunit A,

a 55-130 kDa regulatory subunit B, and a 36 kDa catalytic subunit C. The B regulatory subunits are divided into B, B' and B'' families, which show little homology to each other. These molecules are encoded by many genes and have many splicing variants. The B family proteins, Bα, Bβ, Bγ, and Bδ [24, 25], and the B' family proteins, B'α, B'β, B'γ, B'δ and B'ε [26], are all 54-57 kDa in size. The B'' family consists of PR72/130, PR48 and PR59 [27-29]. A B''' family, consisting of PR93 and PR110, has also been reported recently [30]. At present, a total of 20 B subunit proteins are known, including splicing variants of Bβ, B'γ, B'δ and PR72/130 [11-13]. The diversity of B regulatory subunits is considered to be due to the functional complexity of PP2A, which is involved in several basic phenomena, such as cell cycle regulation [11-13] and tumour progression [21, 31-33]. Studies to identify the substrate specificities and the functions of the various isoforms are currently in progress. Bα regulates the cell cycle through microtubule-associated proteins [34], B'α blocks the Wnt signal, acting on APC [35], and B'γ1 acts on paxillin in focal contacts and regulates cell motility [31]. Recent studies indicated that the B subunit targets PP2A to the MAPK cascade, while the B' subunit regulates apoptosis [36-38]. Thus, it is necessary to analyze the isoforms involved, especially the B isoforms, to understand the signal transduction pathway involved in MAPK-mediated migration.

In the present study, we focused on the role of one isoform, PP2A-Bβ, which has only one B isoform, expressed in a carcinoma cell line. Our results represent evidence for

regulation of MAPK-mediated migration by PP2A-Bβ.

Materials and Methods

Cell culture. A human oesophageal squamous carcinoma cell line, A431, was used (Human Science Research Resource Bank, Osaka, Japan). The cells were maintained as subconfluent cultures in Eagle's minimum essential medium (MEM, Sigma, St. Louis, MO, USA) containing 10% foetal bovine serum (FBS, GIBCO BRL, Grand Island, NY, USA). Cells were starved of serum on 10-cm tissue culture dishes for 18 h in medium containing 1% FBS for 1 day before the experiment. The cells were harvested with 0.25% trypsin, which was then inactivated with 0.25% soybean trypsin inhibitor (Sigma). The cells were washed with serum-free medium containing 0.5% heat-denatured bovine serum albumin (BSA, Sigma), suspended in BSA/MEM, and incubated for 30 min at 37°C for the experiments.

Construction of the antisense expression vector and transfection. A search for homology between PP2A-Bβ and other B subunits was performed (there is poor homology among families of the B regulatory subunit, but high homology within a given B family), and a sequence without high homology was found at positions –10 to +386. There was 70.2% homology between α and β, and 75.8% homology between α and γ isoforms. The sequence of the upstream primer was selected as GCTCTAGACCTGCTGTCAATGGAGGAGGA (*Xba*I recognition site is underlined) and that of the downstream primer was CCCAAGCTTCCTTCTGGCCTCTTATCACGC (*Hin*dIII recognition sequence underlined).

The PCR product was subcloned into pcR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The inserted DNA was sequenced with an autosequencer (Amersham Pharmacia Biotech, Piscataway, NJ, USA), cut out with *Hin*dIII and *Xba*I (Toyobo, Osaka, Japan), and inserted into the mammalian expression vector pcDNA3.1 (Invitrogen), which was linearised at the multicloning site with the same restriction enzymes. The DNA was inserted into pcDNA3.1 in the antisense orientation. The antisense-containing expression vector was transfected into A431 cells using Effectene Transfection Reagent (QIAGEN, Hilden, Germany). Transformed A431 cells were cloned by selection with 400 μg/ml G418 (Sigma). The cells were maintained in 40 μg/ml of G418, and the medium was changed to 1% FBS/MEM without G418 on the day before the experiment. Mock-transfected cells were used as controls.

Reverse transcription (RT)-PCR. Total RNA was extracted from the cells by the acid guanidinium-phenol-chloroform method. Contaminating DNA was removed by incubation with RNase-free DNase I (Nippon Gene, Toyama, Japan) for 30 min at 37°C. The extracted total RNA (1 μg) in a volume of 20 μl was transcribed to cDNA using AMV reverse transcriptase (Promega, Madison, WI, USA) for 10 minutes at 42°C, and then the reaction mixture was heated for 5 minutes to inactivate the enzyme. The resultant cDNA solution (0.5 μl) was used for PCR. The sequences of the primers for detecting isoforms of PP2A B subunits are listed in Table 1. The reactions were performed using KOD-DNA polymerase

containing anti KOD-polymerase (Toyobo), and were run in a thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C/2 min for 1 cycle; 94°C /15 s, 55°C /30 s, 68°C /40 s, for 35 cycles. Amplified DNA was separated on 2% agarose gels containing 2 μg/ml ethidium bromide and visualised under ultraviolet irradiation.

Migration assay. Cell migration assays were performed in 24-well Transwell chambers containing a polycarbonate membrane with a pose size of 8 μm (Costar, Cambridge, MA, USA). The undersurface of the membrane was coated with 10 μg/ml type I COL (Nitta Gelatin, Osaka, Japan) in phosphate-buffered saline at 4°C overnight. Aliquots of 50,000 cells were treated with 10 ng/ml EGF (Genzyme, Cambridge, MA, USA) for 30 min at 37°C. The resuspended cells were washed by centrifugation, loaded onto membranes in quadruplicate, and incubated in a CO_2 incubator at 37°C for 18 to 20 h. The cells that migrated on the COL-coated surface were stained with 2% crystal violet (Sigma). The membranes were washed with water, the dye was eluted with 10% acetic acid, and the optical density of the eluate at 620 nm was measured. The background optical density was evaluated on BSA-coated membranes, and was subtracted from all data points. When the number of migrated cells was too small to apply the colourimetric assay, the cells were counted directly under a microscope.

Western blotting. Cells were lysed with sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 6%

mercaptoethanol), and sonicated. Then, the samples were heated at 95°C for 5 min and centrifuged for 5 min. Supernatants were collected and protein content was determined using a Protein Assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 50 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Perkin-Elmer Life Sciences, Boston, MA, USA). The membranes were reacted with anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) antibodies (New England BioLabs, Beverly, MA, USA) or anti-PP2A- Bα/β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-EPK1/2 antibodies (New England BioLabs) were also used to confirm equal loading of protein samples. The membranes were washed, and then reacted with peroxidase-linked anti-rabbit IgG antibodies (Cell Signaling, Beverly, MA, USA) or peroxidase-linked anti-goat IgG antibodies (Santa Cruz). Visualisation was performed using a chemiluminescence system (ECL western blotting detection reagent, Amersham Pharmacia Biotech). The blots were scanned with a computer-assisted scanner and the band densities were analyzed with NIH Image software. Anti-phospho-ERK1/2, anti-ERK1/2, anti-PP2A-Bα/β, peroxidase-linked anti-rabbit IgG and peroxidase-linked anti-goat antibodies were diluted at 1:1,000, 1:1,000, 1:1,000, 1:2,000 and 1:2,000, respectively. Bα and Bβ proteins could not be distinguished by western blotting using anti-Bα/β antibodies as both have a molecular weight of 55 kDa. However, we regarded the data obtained with anti-Bα/β antibodies as reflecting Bβ protein because Bα mRNA was not detected by highly sensitive

RT-PCR.

Measurement of phosphatase activity. A fluorescence assay method [39] was employed to determine phosphatase activity. Aliquots of 4×10^6 cells were lysed with lysis buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM mercaptoethanol) and sonicated. The samples were centrifuged at 14,000 rpm for 10 min. The protein concentration in the supernatant was measured, and the samples containing the same protein content were used. Polyclonal anti-PP2A-Bα/β antibodies were added to the supernatant, and the mixtures were incubated at 4°C for 90 min. Protein G-Sepharose (Amersham Pharmacia Biotech) was added, and the mixtures were rocked at 4°C for 2-3 h. Protein G-Sepharose with bound immune complexes was washed with lysis buffer several times. The buffer was changed to phosphatase assay buffer (50 mM Tris-HCl buffer, pH 7.0, 0.1 mM $CaCl_2$), and incubation was conducted at 37°C, followed by reaction with 4-methylumbelliferyl phosphate (Wako Pure Chemical Industries, Osaka, Japan), and the fluorescence intensity of the resultant 4-methylumbelliferone was measured with an emission wavelength of 450 nm and excitation wavelength of 360 nm. As a reference, the supernatants of cells were diluted serially beginning with 4×10^6 cells, processed sequentially under the same conditions without reaction with the antibodies, and the fluorescence intensities were plotted. We defined the fluorescence intensity of 4×10^6 cells with 4-methylumbelliferyl

phosphate as 1 artificial unit (AU).

Immunofluorescence. Slide glasses with 8 plastic chambers (Lab-Tek Chamber Slides, Nunc, Roskilde, Denmark) were coated with 10 μg/ml COL. Aliquots of 10,000 cells were loaded onto each well, and incubated at 37°C for 90 min. Cells were fixed with 3% paraformaldehyde for 10 min, permeabilised with 0.01% Triton X-100 for 3 min, and blocked with 2% BSA, and then incubated for 90 min with anti-PP2A-Bβ antibodies (Calbiochem, Darmstadt, Germany) at 1:10 dilution, or anti-PP2A-Bα/β antibodies at 1:10 dilution. The cells were then reacted for 30 min with FITC-conjugated anti-rabbit antibodies (ICN, Costa Mesa, CA, USA) or FITC-conjugated anti-goat antibodies at 1:200 dilution at room temperature. As a negative controls FITC-conjugated anti-rabbit antibodies were reacted with cells without primary antibodies.

Statistics. Student's *t*-test was used for comparison of the averages between two groups. Two-way analysis of variance was used for comparison of kinetic data between two groups.

Results

PP2A-Bβ is Commonly Expressed in Squamous Carcinoma Cell Lines

Twenty isoforms and splicing variants of the PP2A B subunit have been reported [11-13]. We investigated their expression in A431 squamous cell carcinoma cells and other squamous carcinoma cell lines (Calu-1, VMRC-LCP and HSC-3) by RT–PCR. The results showed that Bβ, B'β, B'γ1 and B'δ2 were expressed in A431 cells (Table 1). Furthermore, Bβ

was expressed in all of the squamous carcinoma cell lines examined (Fig. 1). Cell migration of stratified squamous epithelia is a universal phenomenon, occurring for example in wound healing, and it is likely that a particular phosphatase would be involved in regulation of migration among many cells of similar origin. Furthermore, recent studies suggested that B isoforms, but not the B' isoforms, are involved in regulation of the MAPK cascade [36-38]. Therefore, we chose the Bβ regulatory subunit of PP2A to analyse the regulatory mechanism of migration.

Bβ Antisense-transfected Cells Show Decreased Protein Expression and Phosphatase Activity of PP2A-Bβ

Bβ antisense DNA was transfected into A431 cells to inhibit the expression of PP2A-Bβ. To confirm the inhibitory effect, we examined the protein expression level of Bβ by western blotting using anti-PP2A-Bα/β antibodies in three antisense-transfected clones selected at random from ten clones. Three clones of mock-transfected cells were used as controls. Bands at the position of 56 kDa were found in all cell lysates. The density of Bβ in antisense-transfected cells was decreased to 58.8 ± 20.1% (mean ± standard deviation, n=9) of that in mock-transfected cells ($P<0.05$) (Fig. 2A). Next, we measured PP2A- Bβ phosphatase activity by immunoprecipitation using anti-PP2A-Bα/β antibodies. The average phosphatase activity in the mock-transfected cells was 0.224 ± 0.0434 AU, and that in the antisense-transfected cells was 0.108 ± 0.0127 AU. The activity of the antisense-transfected

cells was 48.2% of that of the mock-transfected cells ($P<0.05$) (Fig. 2B). One of the antisense-transfected cell clones was chosen for the following experiments.

Bβ Antisense Decreases EGF- and COL-induced Migratory Activity

To examine the effect of decreased PP2A-Bβ, we performed migration assay using antisense-transfected cells on COL. The time course of migration was examined using one clone each of antisense-transfected (M4) and mock-transfected cells (AS8). The migratory ability of antisense-transfected cells was clearly weakened in both the presence and absence of EGF as compared to mock-transfected controls (Fig. 3A). MTT assay was also performed to determine whether the antisense-transfected cells were viable or not. Microscopically all the cells were adhered to collagen when the cells were loaded onto COL and incubated for 9 hours, and the result of MTT assay [40] showed no difference between antisense-transfected cells and mock-transfected cells (Fig. 3B). Furthermore, the difference in migration in the presence and absence of EGF was larger in the mock-transfected cells than in the antisense-transfected controls (Fig. 3A). Next, we calculated the enhancing effect of EGF on migration for 20 h using 3 clones of antisense-transfected cells as follows: migration with EGF/ migration without EGF (%). As shown in Fig. 3C, antisense transfection reduced the enhancing effect of EGF on migration on COL.

PP2A-Bβ Assembles in Focal Contacts in A431 Cells, but not in Antisense-transfected Cells

After treatment with or without EGF for 30 min, cells were loaded on COL,

incubated in a CO_2 incubator for 90 min, and morphological changes were examined under a microscope. Most of the A431 cells without EGF were round and a few cells were slightly elongated 90 min after adhesion to COL. Following pre-treatment of A431 cells with EGF, many of the cells had cytoplasmic extensions or were polygonal 90 min after adhesion to COL. Antisense-transfected cells without EGF were round on COL. Even when treated with EGF, the antisense-transfected cells did not show elongation of their cytoplasmic extensions and showed little morphological difference from cells treated without EGF.

We further examined the localisation of Bβ protein immunohistochemically. Bβ was not detected in round or polygonal A431 cells on COL. However, Bβ was immunolocalised at the base of short cytoplasmic extensions of EGF-stimulated A431 cells after adhesion to COL (Fig. 4).

MAPK Phosphorylation Stimulated by EGF and/or COL is Decreased in Antisense -transfected Cells

A MAPK cascade activated synergistically by EGF and COL is involved in signal transduction leading to migration of A431 cells [10]. To ascertain whether PP2A-Bβ controls MAPK phosphorylation, we examined the effects of PP2A-Bβ on EGF- and/or COL-induced activation of MAPK. In the absence of EGF, the average band density on western blotting of the mock-transfected cells using anti-phosphorylated MAPK antibodies decreased to 70.2% of that of antisense-transfected controls (Fig. 5A). Next, we examined the response of MAPK

phosphorylation in EGF-stimulated cells. The results indicated that phosphorylation of MAPK after stimulation with EGF was elevated, reaching the maximal level at 15-30 min, and returned to the control level after 60 min, whereas antisense-transfected cells lacked the response to EGF (Fig. 5B, C). No changes were observed in MAPK after stimulation with EGF within the time frame examined (Fig. 5B). To determine the amount of phosphorylated MAPK, samples showing the maximal level of phosphorylated MAPK were diluted serially and subjected to western blotting (Fig. 5B). In the mock-transfected cells, MAPK phosphorylation increased within 15-30 min after adhesion to COL and then returned to the initial level, while the antisense-transfected cells showed no response to COL (Fig. 5D). When cells were treated with EGF for 30 min and then immediately loaded onto plates coated with COL, there was an increase in level of phosphorylated MAPK in the mock-transfected cells within 15 min and this was maintained until 60 min. In contrast, the antisense-transfected cells did not respond as strongly (Fig. 5E).

Weak Effect of EGF and/or COL on PP2A-Bβ Activity in Antisense-transfected Cells

We measured the kinetics of PP2A-Bβ phosphatase activity induced by EGF and/or COL. When mock-transfected cells were treated with EGF, PP2A-Bβ phosphatase activity reached a minimum within 15-30 min and returned to the initial level after 45-60 min (Fig. 6A). After adhesion to COL, PP2A-Bβ phosphatase activity decreased after 30 min, and recovered after 45-60 min in the mock-transfected controls, showing a pattern reciprocal to

that of MAPK (Fig. 6B). After stimulation with both EGF and COL, PP2A-Bβ activity in the mock-transfected cells decreased within 15 min, and maintained the decreased level until 60 min (Fig. 6C). The patterns of the kinetics were reciprocal to the kinetics of MAPK in the mock-transfected cells. That is, EGF activated MAPK and deactivated PP2A-Bβ synchronously. On the other hand, the antisense-transfected cells exhibited low levels of PP2A-Bβ activity and lacked the response of PP2A-Bβ activation to EGF, COL and EGF plus COL (Fig. 6A, B, C). These results also indicated that the antisense-transfected cells, which had low levels of activity of PP2A-Bβ and MAPK, had low migratory activity. Furthermore, the antisense-transfected cells, in which PP2A-Bβ and MAPK activities did not respond to stimulation, showed low migratory responses.

Discussion

Our results indicated that PP2A-Bβ promoted EGF- and COL-induced cell migration. As the EGF-induced migration of A431 cells on COL is regulated by convergent signalling for MAPK activation from EGF receptors and integrins [10], PP2A-Bβ should be associated with MAPK signalling. Indeed, the results of the present study indicated that phosphorylation of MAPK in response to EGF and/or COL was suppressed in Bβ−antisense-transfected cells. Nevertheless, PP2A seems to down-regulate MAPK, because PP2A and MAPK activation have been reported to show an inverse correlation [23, 41]. Furthermore, several recent studies have indicated negative regulation of the MAPK cascade by PP2A. SV40 small T

antigen binds to PP2A and subsequently activates MAPK [17, 42]. PP2A forms a molecular complex with Shc, and regulates Shc tyrosine phosphorylation and downstream mitogenic signalling [43]. In contrast, Bγ has recently been identified as a subunit that promotes neuronal differentiation by activating MAPK [44]. The diversity of isoforms could account for this difference, but PP2A-Bβ activation of mock-transfected A431 cells was inversely correlated with MAPK phosphorylation in the present study. Therefore, the dephosphorylation of a protein involved in the MAPK cascade by PP2A-Bβ should up-regulate MAPK, while phosphorylation should have a down-regulatory effect.

PP2A may interact with MAPK, MEK or Raf-1 in the MAPK cascade. MAPK is activated through phosphorylation of Thr^{202} and Tyr^{204} by MEK [45]. Allessi *et al.* [14] reported that PP2A dephosphorylated Thr^{202} of MAPK. The phosphorylation of Thr^{202} of MAPK is also a signal that is necessary for the transient translocation of MAPK to the nucleus [46]. The signal for migration of A431 cells requires MAPK translocation to the nucleus, as MAPK regulates the production of proteins necessary for migration, including AP-1 [5, 10]. However, dephosphorylation inactivates MAPK. Moreover, Bβ was not localised in the nucleus in the present study. In contrast, Raf-1 has been shown to be localised in the cytoplasm [47], and is membrane-bound following stimulation [48]. It has been reported that PP2A binds directly to Raf-1 and dephosphorylates Raf-1 [49]. Phosphorylation of Ser^{259} inactivates Raf-1, and dephosphorylation of this position by PP2A activates Raf-1

[49]. Thus, Raf-1 or other phosphoproteins upstream of Raf-1, which may be localised at focal contacts, are candidate substrates for PP2A-Bβ.

It has been shown that localisation of PP2A is dependent on the B subunits. Bα colocalises with microtubules [34], B'α, B'β, B'γ1 and B'ε are localised in the cytoplasm and B'γ3 and B'δ are localised in the nucleus [26, 31, 50, 51]. The localisation of Bβ has not been investigated previously. We determined the localisation of PP2A-Bβ by immunofluorescence analysis. Bβ assembled in the cytoplasmic extensions after treatment with EGF on COL. B'γ3 and B'δ1, 2 have a nuclear localisation signal, (K/R)(K/R)XXXXXXXXXXKXXKK [51-53], which mediates their transport to the nucleus [54, 55]. A database search indicated that, with the exception of B'γ3 and B'δ1, 2, the PP2A B subunit proteins (including Bβ) do not have this sequence, supporting the cytoplasmic localisation of Bβ.

The other reported PP2A isoform that is correlated with cell motility is PP2A-B'γ1, which binds to paxillin on focal contacts and inactivates it by dephosphorylation; a defect of B'γ1 has been shown to facilitate cell locomotion [31]. As this isoform, Bγ, was shown to be expressed in A431 cells in the present study, cell migration may be regulated by multiple isoforms. Furthermore, PP2A-Bβ was expressed ubiquitously in the squamous cell carcinoma cell lines A431, HSC-3, VMRC-LCP and Calu-1. Therefore, we concluded that the isoform PP2A-Bβ participates in a common cell motility mechanism involved in the migration of

squamous carcinoma cells.

References

1. Anderson NG, Maller JL, Tonks NK, Sturgill TW: Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature 1990;343:651-653.

2. Matsumoto K, Ziober BL, Yao CC, Kramer RH: Growth factor regulation of integrin-mediated cell motility. Cancer Metastasis Rev 1995;14:205-217.

3. Stoker M, Gherardi E: Regulation of cell movement: the motogenic cytokines. Biochim Biophys Acta 1991;1072:81-102.

4. Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresh DA: Regulation of cell motility by mitogen-activated protein kinase. J Cell Biol 1997;137:481-492.

5. Malliri A, Symons M, Hennigan RF, Hrulstone AF L, Lamb RF, Wheeler T, Ozamne BW: The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells J Cell Biol 1998;143:1087-1099.

6. Hill CS, Treisman R: Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995;80:199-211.

7. Marshall CJ: Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179-185.

8. Chen Q, Lin TH, Der CJ, Juliano RL: Integrin-mediated activation of MEK and

mitogen-activated protein kinase is independent of Ras. J Biol Chem 1996;271:18122-18127.

9. Wary KK, Mainiero F, Isakoff SJ, Marcantonio EE, Giancoti FG: The adapter protein Shc couples a class of integrins to the control of cell cycle progression. Cell 1996;87:733-743.

10. Kawahara E, Nakada N, Hikichi T, Kobayashi J, Nakanishi I: EGF and β1 integrin convergently regulate migration of A431 carcinoma cell through MAP kinase activation. Exp Cell Res 2002;272:84-91.

11. Hunter T: Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 1995;80:225-236.

12. Shenolikar, S: Protein serine/threonine phosphatase - New avenues for cell regulation. Annu Rev Cell Biol 1994;10:55-86.

13. Wera S, Hemmings BA: Serine/threonine protein phosphatases. Biochem J 1995;311:17-29.

14. Alessi DR, Gomez N, Moorhead G, Lewis T, Keyse SM, Cohen SM: Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr Biol 1995;5:283-285.

15. Chung H, Brautigan DL: Protein phosphatase 2A suppresses MAP kinase signaling and protein expression. Cell Signal 1999;11:575-580.

16. Parameswaran N, Nanbi P, Hall CS, Brooks DP, Spielman WS: Adrenomedullin decreases extracellular signal-regulated kinase activity through an increase in protein phosphatase-2A

activity in mesangial cells. Eur J Pharmacol 2000;388:133-138.

17. Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M, Mumby M: The interaction of SV 40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. Cell 1993;75:887-897.

18. Sun H, Charles CH, Lau LF, Tons NK: MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 1993;75:487-493.

19. ZhaoY, Zhang ZY: The mechanism of dephosphorylation of extracellular signal-regulated kinase 2 by mitogen-activated protein kinase phosphatase 3. J Biol Chem 2001;276:32382-32391.

20. Maier GD, Wright MA, Lozano Y, Djordjevic A, Matthews JP, Young MR: Regulation of cytoskeletal organization in tumor cells by protein phosphatases-1 and -2A. Int J Cancer 1995;61:54-61.

21. Ruediger R, Pham HT, Walter G: Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. Oncogene 2001;20:10-15.

22. Young M R, Lozano Y, Djordjevic A, Maier GD: Protein phosphatases limit tumor motility. Int J Cancer 1993;54:1036-1041.

23. Chajry N, Martin PM, Cochet C, Berthois Y: Regulation of p42 mitogen-activated -protein kinase activity by protein phosphatase 2A under condition of growth inhibition by

epidermal growth factor in A431 cells. Eur J Biochem 1996;235:97-102.

24. Hu P, Yu L, Zhang M, Zhao Y, Fu Q, Zhao S: Molecular cloning and mapping of the brain-abundant B1gamma subunit of protein phosphatase 2A, PPP2R2C, to human chromosome 4p16. Genomics 2000;67:83-86.

25. Strack S, Ruediger R, Walter G, Dagda RK, Barwacz CA, Cribbs JT: Protein phosphatase 2A holoenzyme assembly: identification of contacts between B-family regulatory and scaffolding A subunits. J Biol Chem 2002;277:20750-20755.

26. McCright B, Rivers AM, Audlin S, Virshup DM: The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J Biol Chem 1996;271:22081-22089.

27. Hendrix P, Turowski P, Mayer-Jaekel RE, Goris J, Hofsteenge J, Merlevede W, Hemmings BA: Analysis of subunit isoforms in protein phosphatase 2A holoenzyme from rabbit and Xenopus. J Biol Chem 1993;268:7330-7337.

28. Voorhoeve PM, Hijmans EM, Bernards R: Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. Oncogene 1999;18:515-524.

29. Yan Z, Federov SA, Mumby MC, Williams RS: PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with cdc6 and modulates DNA replication in human cells. Mol Cell Biol 2000;20:1021-1029.

30. Moreno CS, Park S, Nelson K, Ashby D, Hubalek F, Lane WS, Palas DC: WD repeat proteins strain and S/G2 nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A. J Biol Chem 2000;275:5257-5263.

31. Ito A, Kataoka TR, Watanabe M, Nishiyama K, Mazaki Y, Sabe H, Kitamura Y, Nojima H: A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. EMBO J 2000;19:562-571.

32. Ruediger R, Walter HT: Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. Oncogene 2001;20:1892-1899.

33. Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J, Evans GA: Alterations of the PPP2R1B gene in human lung and colon cancer. Science 1998;282:284-287.

34. Sontag E, Nunbhakdi-Craig V, Bloom GS, Mumby MC: A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J Cell Biol 1995;128:1131-1144.

35. Seeling JM, Miller JR, Gill R, White R, Virshup DM: Regulation of beta-catenin signaling by B56 subunit of protein phosphatase 2A. Science 1999;283:2089-2091.

36. Silverstein AM, Barrow CA, Davis AJ, Mumby MC: Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. Proc Natl Acad Sci

2002;99:4221-4226.

37. Li X, Scuderi A, Letsou A, Virshup DM: B56-associated protein phosphatase 2A is required for survival and protects from apoptosis in Drosophila melanogaster. Mol Cell Biol 2002;22:3674–3684.

38. Kao G, Tuck S, Baillie D, Sundaram MV: C. elegans SUR-6/PR55 cooperates with LET-92/protein phosphatase 2A and promotes Raf activity independently of inhibitory Akt phosphorylation sites. Development 2004;131:755-765.

39. Cornish CJ, Neale FC, Posen S: An automated fluorometric alkaline phosphatase microassay with 4-methylumbelliferyl phosphate as a substrate. Am J Clin Pathol 1970;53:68-76.

40. Mossman T: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65**:**55-63.

41. Chen J, Parsons S, Brautigan DL: Tyrosine phosphorylation of phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. J Biol Chem 1994;269:7957-7962.

42. Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM: Polyoma small and middle T antigens and SV40 small T antigens form stable complexes with protein phosphatase 2A. Cell 1990;60:167-176.

42. Ugi S, Imamura T, Ricketts W, Olesky JM: Protein phosphatase 2A forms a molecular

complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. Mol Cell Biol 2002;22:2375-2387.

44. Strack S: Overexpression of the protein phosphatase 2A regulatory subunit Bγ promotes neuronal differentiation by activating the MAP kinase (MAPK) cascade. J Biol Chem 2002;277:41525-41532.

45. Payne DM, Rossomando AJ, Martino P, Erickson AK, Her J, Shabanowitz J, Hunt DF, Weber MJ, Sturgill TW: Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J 1991;10:885-892.

46. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J: Nuclear translocation of p42/44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell entry. EMBO J 1999;18:664-674.

47. Mihaly AE, Endrez V, Oravecz T, Rapp UR, Kuhnt U: Immunohistochemical detection of rat protein kinase in cerebral cortical areas of adult guinea pigs and rats. Brain Res 1993;627:225-238.

48. Wartmann M, Hofer P, Turowski P, Saltiel AR, Hynes NE: Negative modulation of membrane localization of the Raf-1 protein kinase by hyperphosphorylation. J Biol Chem 1997;272:3915-3923.

49. Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, Dilworth SM, Mischak H, Kolch W, Baccarini M: Raf-1-associated protein phosphatase 2A as a positive

regulator of kinase activation. J Biol Chem 2000;275:22300-22304.

50. Tanabe O, Gomez GA, Usui Y, Takeda M: Molecular heterogeneity of the cDNA encoding a 74-kDa regulatory subunit (B" or δ) of human protein phosphatase 2A. FEBS Letters 1997;408:52-56.

51. Tehrani MA, Mumby MC, Kamibayashi C: Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. J Biol Chem 1996;271:5154-5170.

52. Csortos C, Zolnierowicz S, Bako E, Durbin SD, DePaoli-Roach AA: High complexity in the expression of the B' subunit of protein phosphatase 2A. Evidence for the existence of at least seven novel isoforms. J Biol Chem 1996;271:2578-2588.

53. Tanabe O, Nagase T, Murakami T, Nozaki H, Usui H, Nishito Y, Hayashi H, Kagamiyama H, Takeda M: Molecular cloning of a 74-kDa regulatory subunit (B" or δ) of human protein phosphatase 2A. FEBS Lett 1996;379:107-111.

54. Dingwall C, Laskey RA: Nuclear targeting sequences - a consensus? Trends Biochem Sci 1991;16:478-481.

55. Robbins J, Dilworth SM, Laskey RA, Dingwall C: Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 1991;64:615-623.

Figure Legends

Fig. 1. Expression of PP2A-Bβ in a variety of squamous carcinoma cell lines, A431, Calu-1, HSC-3 and VMRC, evaluated by RT-PCR. Total RNA was extracted from the cells and reverse transcribed into cDNA, which was then amplified using a primer set for detection of Bβ. The PCR products were electrophoresed on 2% agarose gels.

Fig. 2. Effects of Bβ antisense-transfected A431 cells on protein expression and phosphatase activity. Clones of pcDNA3.1/Bβ antisense-transfected and mock-transfected cells were selected with G418. Three clones of mock-transfected cells (M2, M3, M4) and 3 clones of antisense-transfected cells (AS1, AS8, AS10) selected at random were used. (A) Whole-cell lysates (50 μg of protein) were run in 10% polyacrylamide gel under reducing conditions. Western blotting was performed with anti-Bα/β antibodies. The densities of the bands of protein Bβ appearing at 55 kDa were measured and the results are shown as a bar chart. (B) Phosphatase activity of PP2A-Bβ was measured. Protein immunoprecipitated with anti-PP2A-Bα/β and immobilised Protein G was allowed to react with 4-methylumbelliferyl phosphate, and the fluorescence intensity was measured. Phosphatase activities are expressed in artificial units (AU) and percentages are shown. For details, see Materials and Methods. Each bar shows the mean ± standard deviation of three clones assayed in triplicate (n=9).

Fig. 3. Effects of PP2A-Bβ antisense on cell migration on COL. (A) Antisense-transfected cells (AS8) treated with (open circles) or without EGF (closed circles), and mock-transfected

cells (M4) treated with (open squares) or without EGF (closed squares) were loaded on Transwell chambers, and allowed to migrate on COL for 3, 6 and 9 h. The cells were stained with crystal violet, and the numbers of cells in 5 square areas were counted under a microscope. (B) Viability of cells was examined. Cells were allowed to adhere for 9 hours on a 24-well plate coated with COL. Non-adherent cells were removed and adherent cells were quantified with a MTT assay. (C) Cells loaded on Transwell chambers were allowed to migrate on COL-coated surfaces for 20 h. Migrated cells were stained with crystal violet, eluted with 10% acetic acid, and the absorbance of the eluate at 620 nm was measured. The ratios of migration with/without EGF (%) were calculated, and are plotted in a bar chart. The bar chart and the line chart show means ± standard deviation of four replicate wells.

Fig. 4. Immunofluorescence of Bβ. Cells were treated with or without 10 ng/ml of EGF for 30 min. EGF was washed out, and the cells were loaded onto COL-coated slide glasses. Ninety minutes after adhesion to COL, cells were fixed with 4% paraformaldehyde, permeabilised with Triton X-100 and immunostained with anti-PP2A-Bβ antibodies. Although A431 cells without EGF did not show immunofluorescence (A), PP2A-Bβ was immunolocalised at the base of cytoplasmic extensions of A431 cells treated with EGF in a pattern of focal contacts (B). The results were similar when immunofluorescence analysis was performed using anti-PP2A-Bα/β antibodies.

Fig. 5. MAPK phosphorylation. (A) Three clones of mock-transfected cells (M2, M3 and M4

cells) and 3 clones of antisense-transfected cells (AS1, AS8 and AS10 cells) were lysed. The whole cell lysates were subjected to western blotting using anti-phosphorylated MAPK antibodies. Anti-MAPK antibodies were also used as loading controls for similar amounts of proteins. (B) Antisense-transfected cells (AS8 cells) and mock-transfected cells (M4 cells) were treated with 10 ng/ml EGF and incubated for 15, 30, 45 and 60 min. Cells were lysed and subjected to western blotting using anti-phosphorylated MAPK or anti-MAPK antibodies. Samples diluted to 1/8, 1/4 and 1/2 from the 15-minute EGF stimulation sample were run simultaneously to ensure that the densities of the blots were dependent on the amount of phosphorylated MAPK. (C) The same experiments as shown in B are depicted in a line chart. AS8 cells (closed squares) and M4 cells (open squares) were treated with EGF, and western blotting analysis was performed using anti-phosphorylated MAPK. The densities of the bands were measured. Each bar shows the mean ± standard deviation of triplicate samples. (D) AS8 cells (closed squares) and M4 cells (open squares) were loaded onto COL-coated plastic dishes without EGF. The cells were lysed at 15, 30, 45 and 60 min after loading. (E) AS8 cells (closed squares) and M4 cells (open squares) were treated with 10 ng/ml EGF, loaded immediately onto COL-coated dishes, and incubated for 15, 30, 45 and 60 min. MAPK phosphorylation is shown as the percentage of the density of the phosphorylated MAPK of M4 or AS8 cells in suspension without EGF.

Fig. 6. PP2A-Bβ activities in AS8 cells (closed squares) and M4 cells (open squares).

Phosphatase activities of PP2A-Bβ trimer were measured by fluorescence assay using anti-Bα/β antibodies. (A) Cells were treated with 10 ng/ml EGF and incubated for 15, 30, 45 and 60 min. (B) Cells were loaded onto COL without EGF. The cells were lysed at 15, 30, 45 and 60 min after loading. (C) Cells were treated with 10 ng/ml EGF, loaded immediately onto COL, and incubated for 15, 30, 45 and 60 min. Phosphatase activities are shown as percentages of that of M4 cells without EGF.

Gene	Protein	Primer	Product (bp)	Accession number	A431	Calu-1	VMRC	HSC-3
PPP2R2A	Bα	5'GGAGCTGGAGGAGGGAATGAT3'	236	NM002717	-	-	+	+
		5'TCATGGCTCTGGGAAGGTGCTG3'						
PPP2R2B	Bβ	5'CCAACAGCTATGCGACCGAAG3'	320	NM004576	+	+	+	+
		5'CACGCTCGCTGACTTTCCACA3'						
PPP2R2C	Bγ	5'GAGGACACGGACACGCGGAAAA3'	322	AF086924	-	-	+	-
		5'TGGACAGGAGTGAGTGGGCG3'						
PPP2R5A	B'α	5'GCAAGGCGCAGAGGCAGAAGC3'	374	NM006243	-	-	+	+
		5'GGCCTCAAGCGTGGGGTTCATCC3'						
PPP2R5B	B'β	5'GAGAGGAAGCTGCCCCCTGC3'	288	NM0062244	+	-	-	+
		5'GAGGTCGGCCACACAGTCCAAG3'						
PPP2R5C	B'γ1	5'CCTACGGGAGCGGAATTTGACC3'	994	L42375	+	-	+	-
		5'GCCAAAGCCAATCCCCAGGTAC3'						
	B'γ 2	5'CCTACGGGAGCGGAATTTGACC3'	1041	L42375	-	+	+	+
		5'CTCGGACTTGCGGAGTGCAAGAGG3'						
	B'γ 3		1158	NM002719	-	-	-	-
PPP2R5D	B'δ1	5'GAGAAGGAGCCCCCCAAGGTT3'	585	L76702	-	-	+	-
		5'AGGGTGGGCTCATCTTCCTCTG3'						
	B'δ2		491	L76702	+	-	-	-
PPP2R5E	B'ε	5'CTCAGCACCAACTACTCCTCC3'	391	L76703	-	-	-	-
		5'GCTGTCACTAGGAGGGAGAG3'						
PPP2R3	B''/PR72	5'TTGCCGCTGAGGAGTATGAGAC3'	195	NM002718	-	-	-	-
		5'CCGGCAGCTATTCTTCATCCAC3'						
	B''/PR130		492	NM002718	-	-	-	-

Table 1. A list of primers used for detection of B regulatory subunits of PP2A and the results of RT-PCR. To detect expression of mRNAs of Bβ isoforms or splicing variants, RT-PCR was performed using the primer pairs listed in Table 1. The PCR products were subjected to agarose gel electrophoresis, and bands were visualised with ethidium bromide. A part of the band pattern is shown in Fig. 3.

+, Expression of the mRNA was detected by RT-PCR; –, Expression of the mRNA was not detected.

Fig. 1

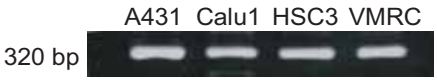


Fig. 2

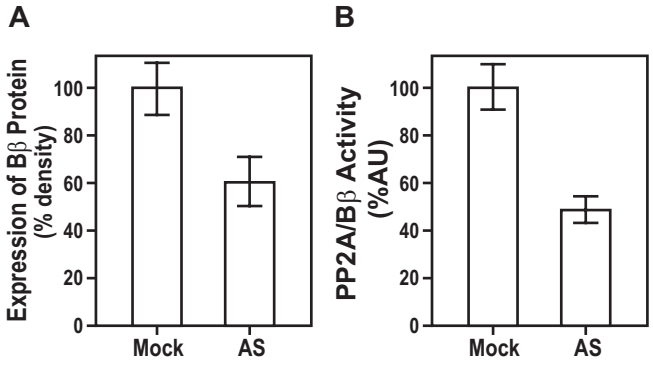


Fig. 3

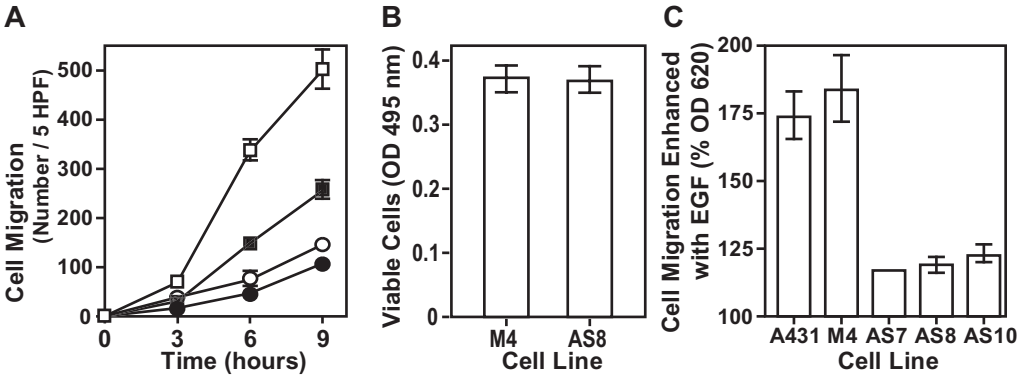


Fig. 4

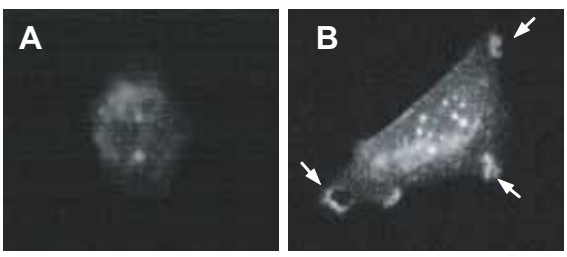


Fig. 5

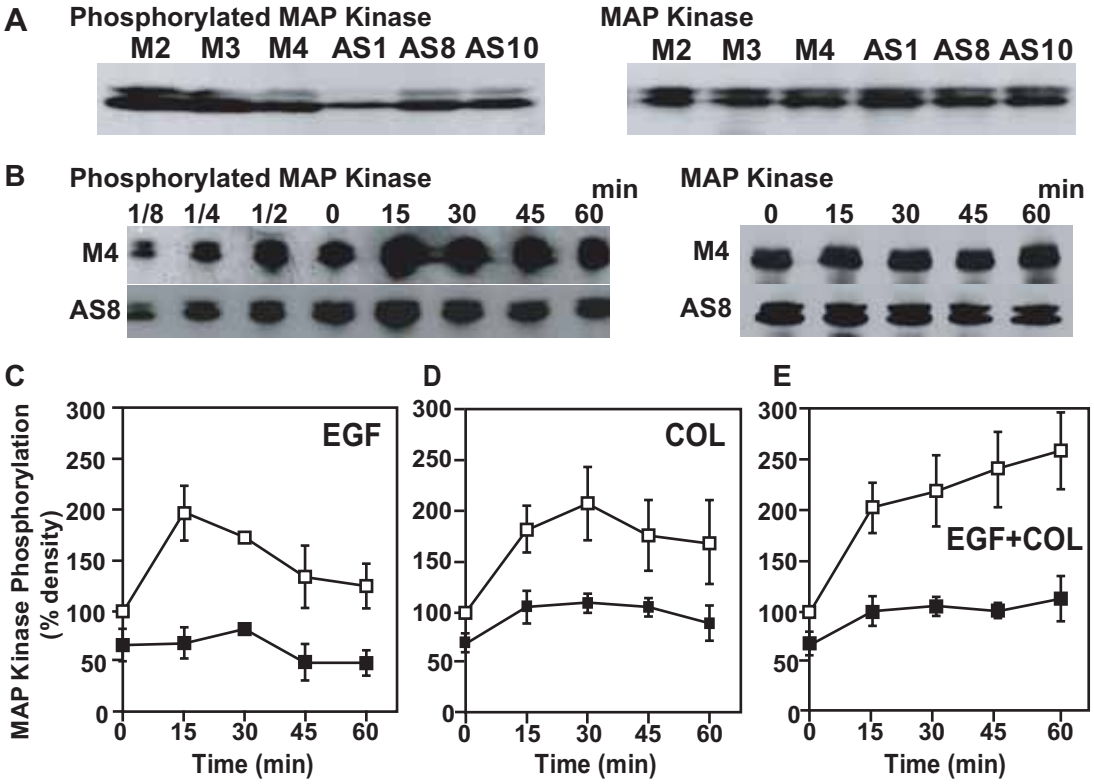
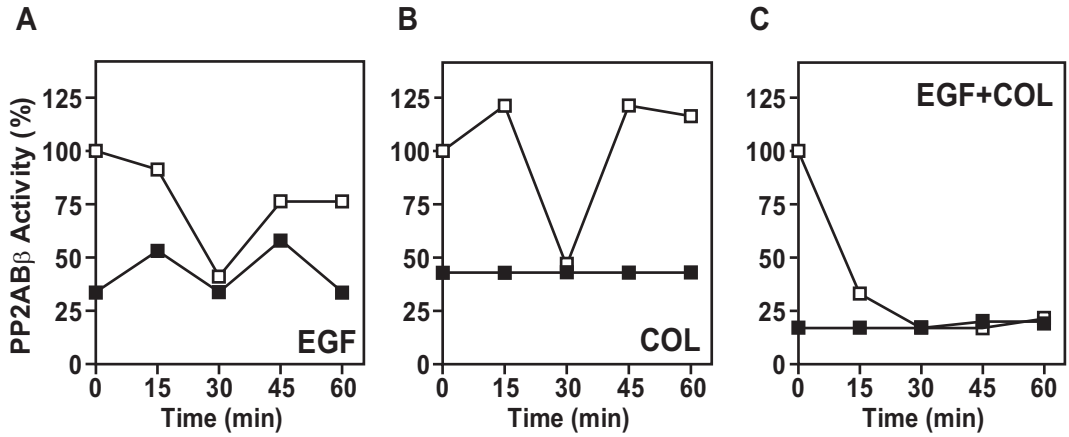

A
Phosphorylated MAP Kinase
M2 M3 M4 AS1 AS8 AS10
MAP Kinase
M2 M3 M4 AS1 AS8 AS10
B
Phosphorylated MAP Kinase
min
1/8 1/4 1/2 0 15 30 45 60
M4
AS8
MAP Kinase
min
0 15 30 45 60
M4
AS8
C
D
E
MAP Kinase Phosphorylation (% density)
EGF
COL
EGF+COL
Time (min)

Fig. 6


A
B
C
PP2ABβ Activity (%)
EGF
COL
EGF+COL
Time (min)