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# 16K Human Prolactin Inhibits Vascular Endothelial Growth Factor-Induced Activation of Ras in Capillary Endothelial Cells

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Signaling pathways mediating the antiangiogenic action of 16K human (h)PRL include inhibition of vascular endothelial growth factor (VEGF)-induced activation of the mitogen-activated protein kinases (MAPK). To determine at which step 16K hPRL acts to inhibit VEGF-induced MAPK activation, we assessed more proximal events in the signaling cascade. 16K hPRL treatment blocked VEGF-induced Raf-1 activation as well as its translocation to the plasma membrane. 16K hPRL indirectly increased cAMP levels; however, the blockade of Raf-1 activation was not dependent on the stimulation of cAMP-dependent protein kinase (PKA), but rather on the inhibition of the GTP-bound Ras. The VEGF-induced tyrosine phosphorylation of the VEGF receptor, Flk-1, and its association with the Shc/Grb2/Ras-GAP (guanosine triphosphatase-activating protein) complex were unaffected by 16K hPRL treatment. In contrast, 16K hPRL prevented the VEGF-induced phosphorylation and dissociation of Sos from Grb2 at 5 min, consistent with inhibition by 16K hPRL of the MEK/MAPK feedback on Sos. The inhibition of Ras activation was paralleled by the increased phosphorylation of 120 kDa proteins comigrating with Ras-GAP. Taken together, these findings show that 16K hPRL inhibits the VEGF-induced Ras activation; this antagonism repre-

sents a novel and potentially important mechanism for the control of angiogenesis. (*Molecular Endocrinology* 13: 692–704, 1999)

## INTRODUCTION

Angiogenesis, the sprouting of new capillaries from the preexistent blood vessels, is of central importance in many biological processes, including embryonic vascular development and differentiation, wound healing, and organ regeneration (1). In addition, angiogenesis plays a major role in pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases, tumor growth, and metastasis (2). During angiogenesis, endothelial cells migrate, proliferate, organize into tube-like structures, and play an active role in tissue remodeling. This cascade of events is under the control of angiogenic factors (reviewed in Ref. 1), which include basic fibroblast growth factor (bFGF) (3) and vascular endothelial growth factor (VEGF) (4) or vascular permeability factor (VPF) (5), an endothelial-cell specific mitogen. *In vivo*, both factors have been shown to stimulate the formation of new capillary beds (reviewed in Ref. 1). *In vitro*, both factors stimulate the proliferation of vascular endothelial cells from a variety of sources including bovine brain capillary endothelial cells (BBE) (6, 7). In addition to positively acting angiogenic factors, there appears to be an equally important series of factors

that inhibit angiogenesis (reviewed in Ref. 1). Inhibitors of angiogenesis include thrombospondin (8), platelet factor 4 (9), 16K hPRL (7), angiostatin (10), and endostatin (11). Antiangiogenic factors have been shown to antagonize both the proliferation of endothelial cells *in vitro* and neovascularization *in vivo* (reviewed in Ref. 1).

The actions of both bFGF and VEGF are mediated by receptors of the tyrosine kinase family (12, 13). One of the potential pathways leading from tyrosine kinase receptors to the activation of the mitogen-activated protein kinase (MAPK) involves Shc-Grb2-Sos-Ras-Raf-MEK-MAPK. Binding of agonists results in 1) receptor dimerization, 2) activation of a tyrosine kinase, 3) autophosphorylation of the receptor, and 4) recruitment of adapter molecules via Src homology domains (SH2) such as Shc or Grb2 (14, 15). Grb2, through its SH2 domain, can associate with the tyrosine-phosphorylated Shc (16) or can bind directly to growth factor receptors and mediate, through its SH3 domains, the recruitment of *Son of sevenless* (Sos) to the membrane (17) and activates Ras. Ras, located at the plasma membrane, associates with and stimulates the serine/threonine kinase Raf-1, which phosphorylates and activates the dual-specificity threonine/tyrosine kinase MEK (MAPK kinase). MEK, in turn, phosphorylates p44 and p42 MAPK, resulting in increased expression of early response genes and stimulation of cell proliferation. In contrast, Ras inactivation is regulated by a Ras-guanosine triphosphatase (GTPase)-activating protein (Ras-GAP), which stimulates the hydrolysis of GTP-bound Ras to a GDP-bound state (18). The balance between the activities of Sos and Ras-GAP regulates the activation state of Ras.

These signaling pathways have been partially confirmed for the action of VEGF. In porcine aortic endothelial cells overexpressing KDR, the human homolog of Flk-1, occupancy of the receptor promoted the association and phosphorylation of Shc and the induction of Shc-Grb2 complex formation (19). In aortic endothelial cells, treatment with VEGF resulted in tyrosine phosphorylation of several proteins including Ras-GAP (20). We previously reported, in BBE cells, that VEGF activated Raf-1 (21) and MAPK (22).

Signaling mechanisms that turn off cell proliferation include an increase in cAMP levels, a process mediated through the activation of cAMP-dependent protein kinase A (PKA) (23, 24). Consistent with previous observations (23–25), we demonstrated that, in BBE cells, PKA blocked VEGF-induced MAPK pathway at the level of Raf-1 (21). We also showed that 16K PRL inhibited the mitogenic action of both bFGF and VEGF on BBE cells (6, 7). Currently, the signaling pathway for the antiproliferative action of 16K hPRL has been partially described (22). A high-affinity membrane receptor that was neither the PRL, bFGF, nor VEGF receptor was identified by ligand binding studies (26). In addition, we previously demonstrated that 16K hPRL inhibited VEGF-induced phosphorylation and activation

of MAPK by acting downstream to the autophosphorylation of Flk-1 (22).

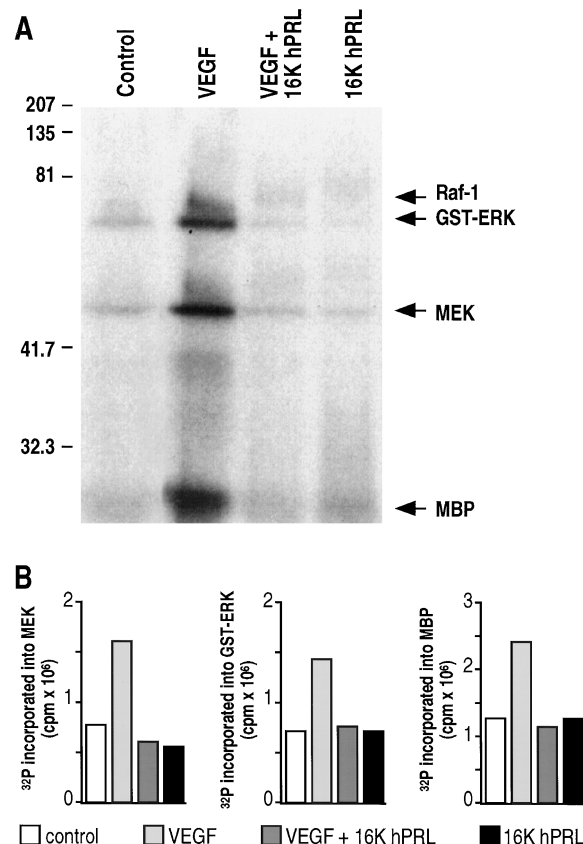
In the highly differentiated BBE cells, we now show that 16K hPRL blocks VEGF-induced Raf-1 activity. The blockade of Raf-1 by 16K hPRL was not dependent on the increase of cAMP levels and the subsequent activation of PKA, but rather on the inhibition of the GTP-bound form of Ras. Consistent with the inactivation of Ras, translocation of Raf-1 to the plasma membrane was blocked as shown by cell fractionation and immunofluorescence studies. The negative effect of 16K hPRL on the VEGF-induced activation of Ras did not involve an upstream signaling event including tyrosine phosphorylation of Flk-1 or association of the activated Flk-1 with Shc/Grb2/Ras-GAP complexes. The inhibition of Ras by 16K hPRL was correlated with a further increase in phosphorylation of Flk-1-associated 120-kDa proteins comigrating with Ras-GAP. These findings represent a potential mechanism for the inhibition of the MAPK signaling cascade activated by VEGF.

## RESULTS

### 16K hPRL Inhibits VEGF-Induced Raf-1 Activity

We have previously shown that 16K hPRL inhibits both bFGF and VEGF-induced MAPK activity (22). In this report we investigated whether the blockade of MAPK activation was at a step more proximal in the signaling cascade, *i.e.* at the level of Raf-1. Raf-1 activity was evaluated by an *in vitro* linked kinase assay in which immunoprecipitated Raf-1 from plasma membrane and cytosolic fractions was incubated with three exogenous proteins, recombinant MEK, GST-ERK (MAPK), and myelin-basic protein (MBP), the substrates of Raf-1, MEK, and MAPK, respectively (27, 28). We verified by Western blotting using the anti-Raf-1 antibody that each immunoprecipitated contained the same amount of Raf-1 (data not shown). Recombinant MEK and GST-ERK were added in excess to ensure that the activity of the immunoprecipitated Raf-1 was limiting (28). Consistent with previous observations (27, 28), Raf-1 immunoprecipitates from cytosolic fractions were not contaminated with MEK or MAPK, since no phosphorylation of MBP was observed when the recombinant enzymes were omitted from the assay (data not shown).

Treatment of the plasma membrane fractions with VEGF induced a similar 2-fold stimulation, over the control levels, of the phosphorylation of the substrates for the endogenous Raf-1 and exogenous MEK and GST-ERK (Fig. 1, A and B). A potent inhibitory effect of 16K hPRL was observed on the VEGF-induced activation of Raf-1 and the distal kinases in the signaling cascade; indeed, after addition of 16K hPRL, Raf-1, MEK, and MAPK activities were totally inhibited and returned to basal levels (Fig. 1, A and B). VEGF also induced a 7-fold increase of Raf-1 phosphorylation,



**Fig. 1.** Inhibition of VEGF-Induced Activation of Raf-1 in Detergent-Solubilized Membrane Fractions by 16K hPRL

Salt-washed membrane fractions (P100) prepared from BBE cells untreated or stimulated for 5 min with 1 nM VEGF, 1 nM 16K hPRL alone, or in combination with VEGF were solubilized in lysis buffer containing 1% NP40. A, Proteins were immunoprecipitated with an anti-Raf-1 polyclonal antiserum, and Raf-1 kinase activity was assessed by an *in vitro* linked kinase assay. Samples were resolved by SDS-PAGE (12% gels) and visualized by autoradiography. Molecular weights are indicated on the left. B, The radioactivity incorporated into MEK, GST-ERK/MAPK, and MBP was evaluated by phosphorimaging and represents Raf-1, MEK, and MAPK activities, respectively. Data shown are from a single experiment representative of three that gave similar results.

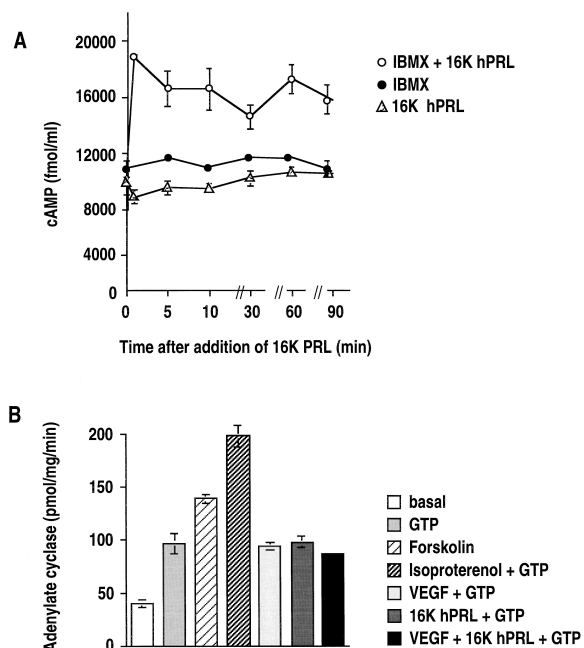
which was completely blocked by 16K hPRL (Fig. 1A). The finding that inhibition of phosphorylation of Raf-1 is greater than the inhibition of kinase activity suggests that all of the phosphorylated residue(s) on Raf-1 may not be involved in the Raf-1 kinase activity.

#### Activation of PKA Is Not Necessary for the Inhibition of Raf-1 by 16K hPRL

Several reports have demonstrated that the inhibition of Raf-1 activity by increased cAMP levels is mediated via the activation of PKA (23–25). In BBE cells we recently showed that increased intracellular levels of cAMP, via the activation of PKA, blocked VEGF-in-

duced Raf-1 activation (21). Treatment of BBE cells with 1 nM 16K hPRL resulted in a rapid and prolonged increase in cAMP levels consistent with the idea that Raf-1 activation was blocked by activation of PKA (Fig. 2A). The experiment was performed in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), to prevent the rapid hydrolysis of cAMP permitting the detection of the increase (21). We then asked whether the increase in the rate of cAMP formation was mediated via the activation of membrane-associated adenylyl cyclase (AC). Addition of forskolin, which directly stimulates AC, or isoproterenol (ISO), a  $\beta$ -adrenergic agonist activating  $\beta$ 2 receptors expressed on BBE cells, stimulated AC activity in BBE cell membranes. However, treatment with 16K hPRL had no effect, suggesting that the stimulation of cAMP levels was mediated by an unknown indirect mechanism (Fig. 2B).

The increase in cAMP levels by 16K hPRL did not appear to be essential for the blockade of the VEGF-induced Raf-1 activity. As above, Raf-1 activity was measured in an *in vitro* assay using recombinant MEK



**Fig. 2.** The Effect of 16K hPRL on Intracellular cAMP Levels and on AC Activity

A, Time-course of stimulation by 16K hPRL of cAMP accumulation in BBE cells. Serum-starved BBE cells were cultured for 90 min in the presence or absence of IBMX (0.5 mM). At the indicated time points, 16K hPRL (1 nM) was added. Levels of cAMP were determined by RIA. Values represent the mean  $\pm$  SEM of triplicates. Similar results were obtained in four independent experiments. B, BBE cell plasma membrane preparations were left untreated or treated with different agents as follow: 100  $\mu$ M forskolin, 50  $\mu$ M GTP, 100  $\mu$ M ISO, 1 nM 16K hPRL, and 1 nM VEGF. Values for AC activity are the mean  $\pm$  SD of triplicates. Similar results were obtained in three independent experiments.



as a substrate. Treatment with 1, 10, or 50  $\mu\text{M}$  H89, a specific inhibitor of PKA (29), did not block the inhibitory effect of 16K hPRL on the VEGF-stimulated Raf-1 activation (data not shown). This finding was confirmed at the level of VEGF-induced MAPK activation by an *in vitro* assay with immunoprecipitated MAPK (data not shown). However, the possibility exists that the cAMP signaling pathway may represent a redundant mechanism by which 16K hPRL could suppress Raf-1 activation.

### 16K hPRL Prevents VEGF-Induced Raf-1 Translocation to the Plasma Membrane

It has been recently demonstrated that an essential step in activation of Raf-1 is its translocation to the plasma membrane (27, 30, 31). To determine whether VEGF and 16K hPRL could modulate the subcellular localization of Raf-1, normalized protein content in the cytosolic (S100) and plasma membrane (P100) fractions were separated by electrophoresis, and the presence of Raf-1 was detected by protein immunoblotting. Figure 4A clearly shows that in untreated cells, the majority of Raf-1 was in the cytosolic fraction; only 10% of the protein was detected in the plasma membrane fraction. In VEGF-stimulated cells, 50% of Raf-1 was translocated to the plasma membrane (Fig. 3A). In contrast, Raf-1 was more abundant in the cytosolic fraction from cells cotreated with VEGF and 16K hPRL. The addition of 16K hPRL dramatically inhibited (75%) VEGF-induced Raf-1 translocation compared with unstimulated cells. In cells treated with 16K hPRL alone, as in control cells, Raf-1 was more abundant in the cytosolic fraction. Similar results were also obtained in BBE cells stimulated with bFGF in the presence or absence of 16K hPRL (data not shown).

These observations were confirmed by the cellular localization of Raf-1 by immunofluorescence, as shown in Fig. 3B. After VEGF treatment, Raf-1 was translocated to the plasma membrane in what appears to be a clustered pattern consistent with receptor capping (Fig. 3B, panel b). After treatment with both VEGF and 16K hPRL, translocation of Raf-1 to the plasma membrane was substantially blocked as was the clustering of the receptors (Fig. 3B, panel c). In contrast, in control cells (Fig. 3B, panel a) or cells treated with 16K hPRL alone (Fig. 3B, panel d), Raf-1 was distributed throughout the cytoplasm.

### 16K hPRL Inhibits VEGF-Activated Ras

Several recent reports have strongly suggested that the principal function of activated Ras in Raf-1 activation is the recruitment of Raf-1 to the plasma membrane (for review, see Ref. 32). Therefore, because we observed a blockade of VEGF-induced Raf-1 translocation to the plasma membrane in the presence of 16K hPRL, we next studied the effect of VEGF and 16K hPRL on Ras activation in BBE cells (Fig. 4, A and B). In the absence of VEGF, a small fraction (20%) of Ras

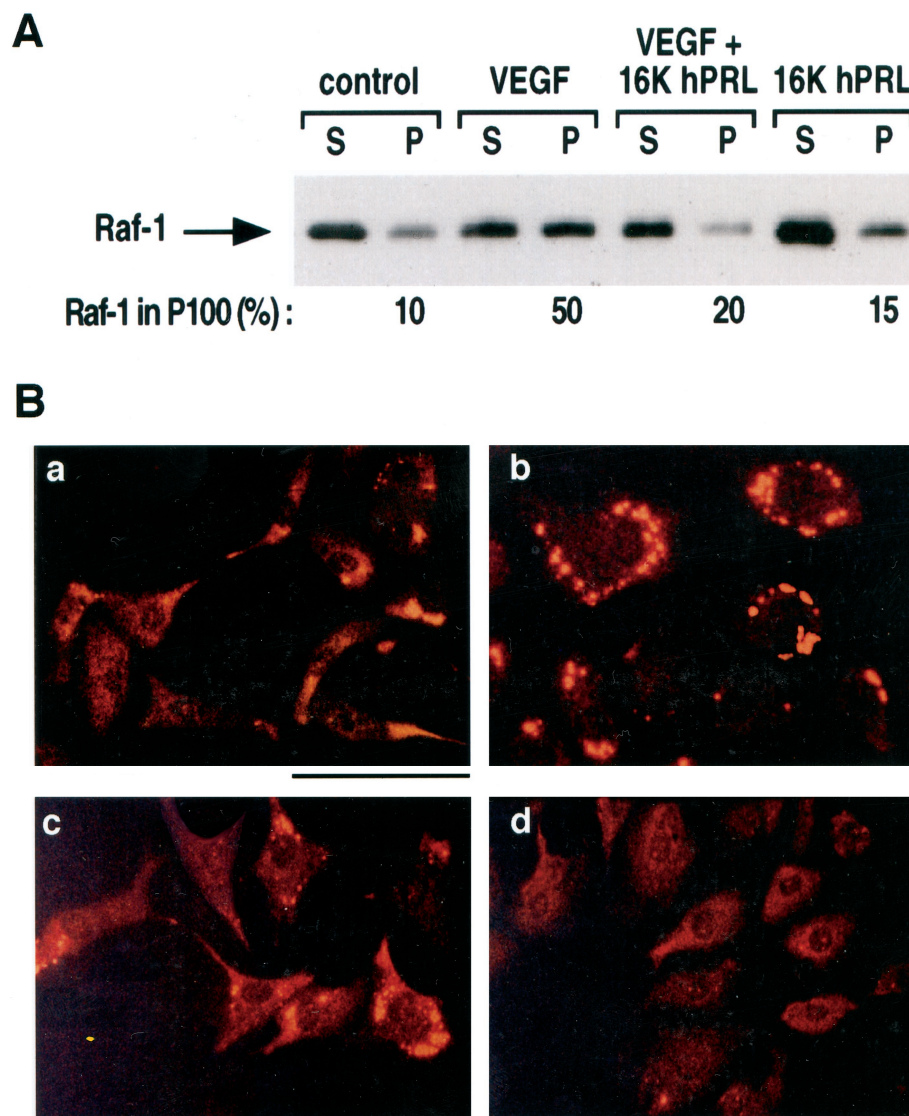
was in the GTP-bound state. Treatment with VEGF stimulated a 3-fold increase in the amount of GTP bound to Ras. The addition of 16K hPRL reduced the increase in the Ras GTP-bound state stimulated by VEGF by nearly 80%, while 16K hPRL alone had no effect (Fig. 4B). Thus, the inhibitory effect of 16K hPRL on VEGF-induced conversion of Ras to the activated GTP-bound state correlates with the blockade of Raf-1 translocation by 16K hPRL.

### Effect of 16K hPRL Upstream of Ras Activation

We have previously demonstrated that 16K hPRL had no effect on VEGF-induced tyrosine phosphorylation of Flk-1 on BBE cells (22). To investigate the effect of 16K hPRL on proteins that link Flk-1 to the Ras cascade, BBE cell lysates were immunoprecipitated with an anti-Flk-1 antibody. The first half of Flk-1-immunoprecipitated proteins was separated by SDS-PAGE and immunoblotted with an antiphosphotyrosine antibody. As shown in Fig. 5A, VEGF treatment induced the tyrosine phosphorylation of several Flk-1-associated proteins including proteins migrating at 200, 160, 120, 66, 52, 46, and 44 kDa. Consistent with our previous observations, addition of 16K hPRL specifically inhibited the VEGF-induced tyrosine phosphorylation of the 44 kDa protein without affecting the phosphorylation level of the 160-kDa protein, identified as MAPK and phospholipase C- $\gamma$ , respectively (22).

The remaining half of Flk-1 immunoprecipitations was probed with different specific antibodies to identify Flk-1-associated proteins. As observed in Fig. 5B, the anti-Flk-1 polyclonal antiserum recognized the 200-kDa protein and confirmed equal loading of the gel. VEGF treatment induced the phosphorylation of proteins migrating at 120 kDa. Interestingly, treatment with 16K hPRL further increased the VEGF-induced tyrosine phosphorylation (Fig. 5A). We identified Ras-GAP as one of these proteins and observed that it was constitutively associated with Flk-1 (Fig. 5C). Furthermore, additional Ras-GAP associated with Flk-1 was recruited (2.5-fold) in a ligand-dependent manner. The cotreatment with 16K hPRL consistently diminished this association to a small extent.

The immunoprecipitation with anti-Flk-1 polyclonal antiserum followed by immunodetection with an anti-Shc monoclonal antiserum revealed that the three Shc isoforms of 46, 52, and 66 kDa were constitutively associated with Flk-1 (Fig. 5D). Although the association of the Shc isoforms with Flk-1 was only slightly increased by VEGF, VEGF dramatically stimulated the tyrosine phosphorylation of the proteins (Fig. 5, A and D). Phosphorylation of the 46- and 52-kDa isoforms of Shc protein was previously reported in porcine aortic endothelial cells overexpressing KDR (19). VEGF-induced phosphorylation or association of Shc isoforms with Flk-1 was not affected by addition of 16K hPRL (Fig. 5, A and D). 16K hPRL alone had no effect on the



**Fig. 3.** Inhibition by 16K hPRL of VEGF-Induced Raf-1 Translocation to the Plasma Membrane

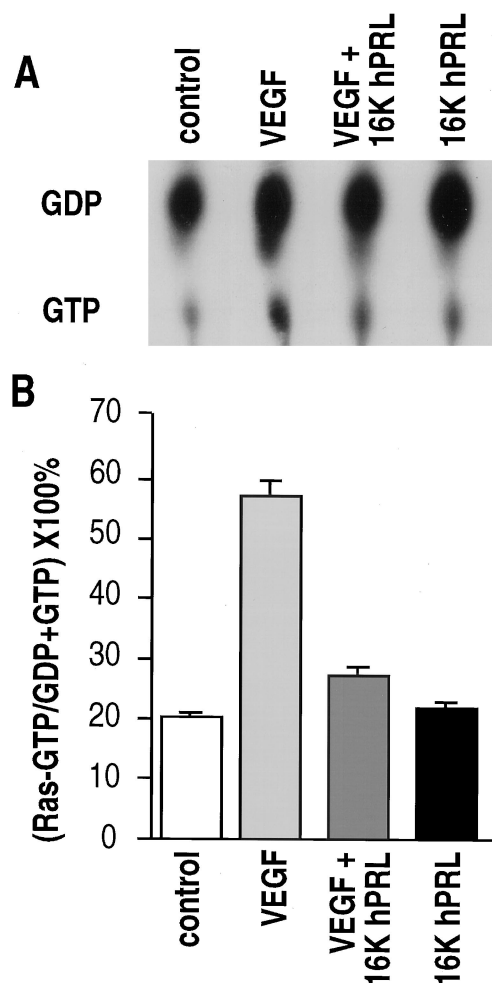
**A**, Cytosolic (S100) and plasma membrane (P100) fractions from BBE cells untreated or stimulated for 5 min with 1 nM VEGF, 1 nM VEGF plus 1 nM 16K hPRL, or 1 nM 16K hPRL alone were normalized for total protein content and immunoprecipitated with anti-Raf-1 polyclonal antiserum. Immunocomplexes were resolved by SDS-PAGE (12% gel), transferred to nitrocellulose membranes, and blotted with anti-Raf-1 polyclonal antiserum. Bands were visualized with an ECL kit. The translocation of Raf-1 to the plasma membrane was determined by densitometry and is expressed as percent of the total Raf-1 in S100 and P100 for each tested condition. Similar results were observed in three additional experiments. **B**, BBE cells were left untreated (panel a) or stimulated with 1 nM VEGF (panel b), 1 nM VEGF plus 1 nM 16K hPRL (panel c) or 1 nM 16K hPRL alone (panel d). Raf-1 was immunolocalized using an anti-Raf polyclonal antiserum and stained with rhodamine-donkey antirabbit Ig (panels a, c, d, magnification,  $\times 20$ ; panel b, magnification,  $\times 40$ ; scale bar under a is 100  $\mu\text{m}$  for panels a, c, d and 50  $\mu\text{m}$  for panel b).

association of the three Shc isoforms with Flk-1 (Fig. 5D). Interestingly, the 46-kDa Shc isoform appeared to be phosphorylated and associated to Flk-1 even in the absence of ligand (Fig. 5, A and D).

Stimulation with VEGF resulted in the association of Grb2 with Flk-1 as shown in Fig. 5E. This association might occur via the binding of Grb2 to the phosphotyrosines on Flk-1 or those on the 52- and 66-kDa isoforms of Shc. The binding of Grb2 with Flk-1 was not modified by the addition of 16K hPRL (Fig. 5E).

#### Effect of VEGF and 16K hPRL on Grb2-Sos Complex

In many types of unstimulated cells, the SH3 domains of Grb2 bind to the proline-rich carboxy-terminal domain of Sos (33, 34). We therefore asked whether Grb2 and Sos were associated in BBE cells and whether this association was affected by VEGF and/or 16K hPRL treatment. In the absence of any treatment, immunoprecipitation of BBE cell lysates with a Grb2 polyclonal



**Fig. 4.** Inhibitory Effect of 16K hPRL on VEGF-Induced Ras Activation

A, Quiescent BBE cells were labeled with [ $^{32}$ P]orthophosphate before stimulation with 1 nM VEGF, 1 nM VEGF plus 16K hPRL, or 1 nM 16K hPRL for 5 min. After lysis, samples were immunoprecipitated with anti-Ras monoclonal antibody, and bound GTP and GDP were resolved on polyethyleneimine-cellulose plates by TLC and quantitated. B, The proportion of radioactivity in GTP and GDP is expressed as  $(2/3 \times \text{GTP}/\text{GDP} + 2/3 \times \text{GTP}) \times 100$ . The means and SES of three independent experiments are shown.

antibody resulted in the coimmunoprecipitation of Sos as revealed by Western blotting using a Sos polyclonal antibody (Fig. 6A). Proteins were equally loaded in all four groups (Fig. 6A, lower panel). Five minutes after VEGF stimulation, a 30% decrease in Grb2-immunoprecipitated Sos protein was observed (Fig. 6A). The simultaneous treatment with 16K hPRL prevented a VEGF effect on Grb2-Sos dissociation. 16K hPRL alone had no effect on Grb2-Sos complex compared with the control (Fig. 6A).

The uncoupling of Grb2-Sos interactions has been attributed to an increase of the MAPK-dependent phosphorylation of Sos as a negative feedback mechanism (35). To determine whether the phosphorylation

of Sos was a consequence of VEGF-induced MAPK activity, we examined, in BBE cells, the effect of VEGF treatment on the SDS-PAGE mobility of Sos. As shown in Fig. 6B, VEGF stimulation of BBE cells resulted in a clear decrease in the electrophoretic mobility of Sos. As expected, the addition of 16K hPRL prevented the VEGF effect on Sos phosphorylation. These results are consistent with the ability of 16K hPRL to inhibit VEGF-stimulated MAPK activity (22). 16K hPRL alone had no effect as compared with the control.

## DISCUSSION

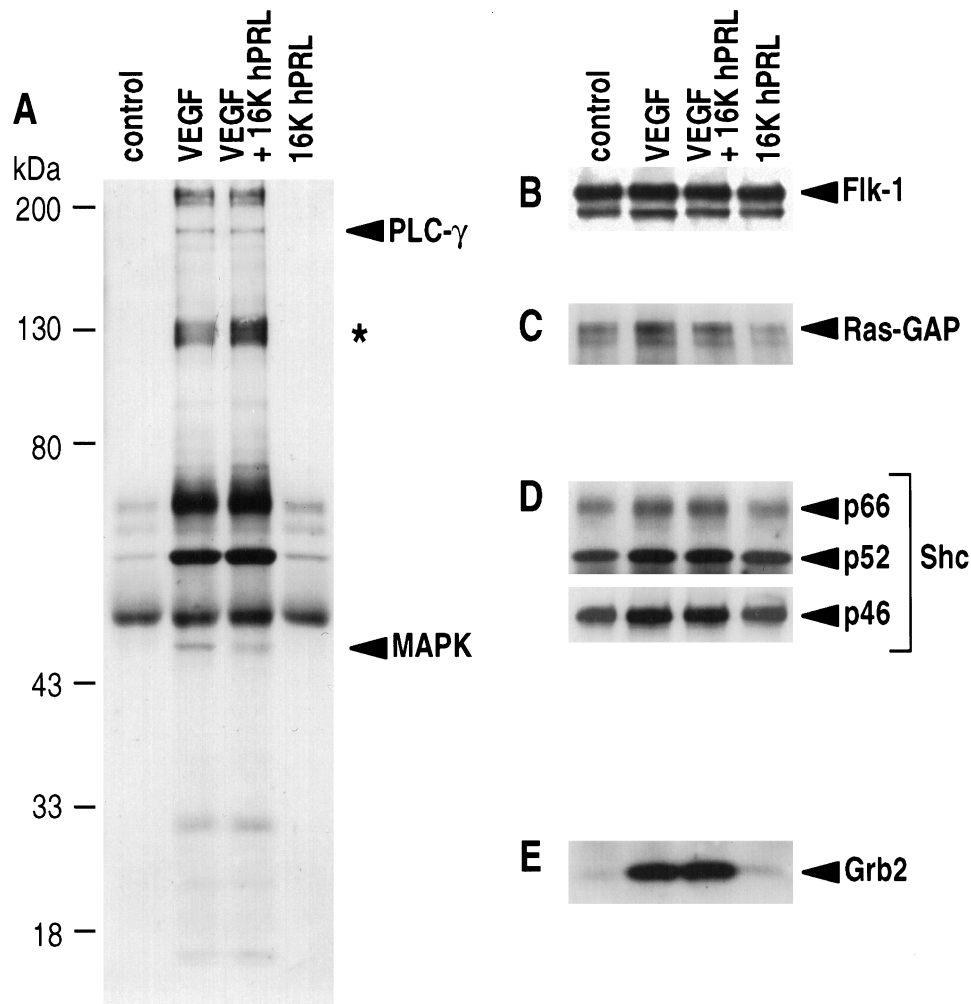
We have previously shown that in BBE cells VEGF and bFGF signal via activation of the MAPK pathway. The antiangiogenic factor 16K hPRL inhibits the activation of MAPK by acting downstream to the autophosphorylation of the VEGF and bFGF receptors (22) (Fig. 7). The aim of the present study was to determine whether the inhibition of MAPK by 16K hPRL occurred at a more proximal step in the signaling cascade.

In a recent report we demonstrated that, in BBE cells, treatment with VEGF or bFGF promoted a rapid activation of the serine-threonine kinase Raf-1 (21). Therefore, we asked whether Raf-1 plays a role in the inhibitory action of 16K hPRL on mitogen-induced activation of MAPK. Using an *in vitro* linked kinase assay (28), we found that VEGF enhanced Raf-1, MEK, and MAPK activities in a linear manner, resulting in a doubling of the activity of each protein. The 2-fold VEGF activation of the MAPK cascade correlates with the stimulation of BBE cell proliferation (7). The blockade of the VEGF-induced MAPK activation by 16K hPRL was due to the inhibition of Raf-1, since 16K hPRL treatment resulted in the complete inhibition of VEGF-induced Raf-1/MEK/MAPK activities.

We have recently found that in BBE cells, as in other cell types (23–25), high levels of cAMP block mitogen-induced Raf-1 activation (21). We also provided evidence that increased levels of cAMP mimic the inhibitory effect of 16K hPRL on mitogen-induced BBE cell proliferation and activation of Raf-1 (21). Treatment with 16K hPRL increased cAMP levels in a time-dependent manner; however, this action does not appear to involve a direct activation of AC. Although 16K hPRL stimulated intracellular levels of cAMP, the assumed activation of PKA did not appear to play a major role in the suppression of VEGF-induced Raf-1 activity. This conclusion is based on the finding that treatment with H89 failed to reverse the action of 16K hPRL. However, the possibility exists for pleiotropy in the signaling pathway which, under certain circumstances, permits activation of PKA to participate in the inhibitory action of 16K hPRL (Fig. 7).

Because Raf-1 activation depends on its recruitment to the plasma membrane by activated Ras (27,





**Fig. 5.** Effect of 16K hPRL Upstream of Ras

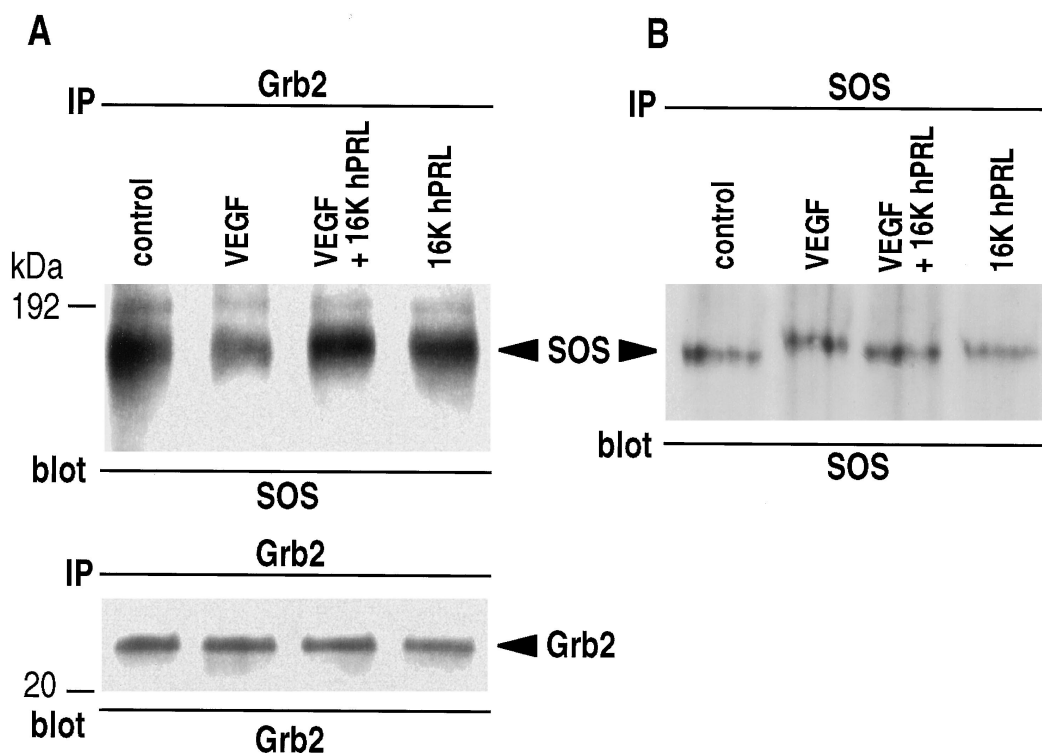
Quiescent BBE cells were left untreated or were stimulated for 5 min with 1 nM VEGF, 1 nM VEGF plus 1 nM 16K hPRL, or 1 nM 16K hPRL. Cell extracts were immunoprecipitated with an anti-Flk-1 polyclonal antiserum, resolved by SDS-PAGE (8% gels), transferred to Immobilon-P, and probed with an antiphosphotyrosine antibody (A) or with an anti-Flk-1 polyclonal antiserum (B). The blot in panel B was stripped before immunodetection with an anti-Ras-GAP monoclonal antiserum (C) or an anti-Shc monoclonal antiserum (D). Two different exposure times were used to visualize p46 isoform before saturation and p52 and p66 isoforms after longer exposure. E, Immunoblot in panel B was stripped and reprobed with an anti-Grb2 monoclonal antiserum. Proteins of interest (arrows) are indicated. Panel B also shows that immunoprecipitated proteins were evenly loaded. Molecular weights are shown on the left side of panel A. Note an increase in the level of tyrosine-phosphorylated proteins (\*). Data shown are from a single experiment representative of four that gave similar results.

36), we examined the effect of VEGF and 16K hPRL on Raf-1 subcellular localization. The current data are the first to show that stimulation with VEGF resulted in the translocation of Raf-1 to the plasma membrane in BBE cells. Importantly, VEGF-induced translocation of Raf-1 was inhibited by 75% after 16K hPRL treatment. Thus, the inhibitory effect of 16K hPRL on Raf-1 translocation was consistent with the blockade of Ras activation. Indeed, the stimulation of Ras activity by VEGF was suppressed by 16K hPRL.

Upstream of Ras, activation of tyrosine kinase receptors rapidly leads to the phosphorylation of Shc adapter proteins (Fig. 7). In BBE cells, the three isoforms of Shc were constitutively associated with Flk-1.

VEGF-induced activation of Flk-1 resulted in a modest recruitment of Shc to the receptor and in a major increase in tyrosine phosphorylation of the 52- and 66-kDa isoforms of Shc. Furthermore, the association of Grb2 with Flk-1 was totally dependent on VEGF stimulation. This association might be the result of interactions between the SH2 domain of Grb2 and the phosphotyrosine residues on Flk-1 and/or on the 52- and 66-kDa Shc isoforms. Similar results were recently reported in porcine aortic endothelial cells overexpressing KDR (19); however, the constitutive association of Shc isoforms to Flk-1 appears to be unique to BBE cells. In addition, our data showed that treatment with 16K hPRL did not affect the VEGF-dependent





**Fig. 6.** Effect of VEGF and 16K hPRL on Grb2/Sos Complex

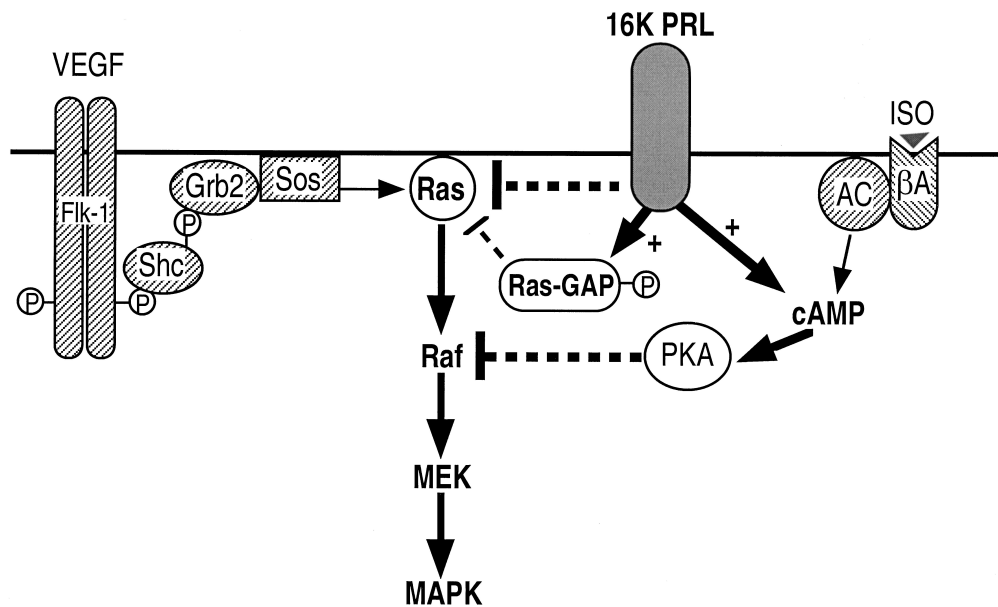
Quiescent BBE cells were left untreated or were stimulated for 5 min with 1 nM VEGF, 1 nM VEGF plus 1 nM 16K hPRL, or 1 nM 16K hPRL. A, Cell extracts were immunoprecipitated (IP) with an anti-Grb2 polyclonal antibody, resolved by SDS-PAGE (7.5%), transferred to Immobilon-P, and probed with an anti-Sos antibody (*top panel*) or an anti-Grb2 antibody after stripping (*bottom panel*). B, Cell extracts were immunoprecipitated with an anti-Sos polyclonal antibody, resolved by SDS-PAGE (6%), transferred to Immobilon-P, and probed with an anti-Sos antibody. Proteins of interest (*arrows*) are indicated. Molecular weights are indicated on the *left* side of panel A. Data shown are from a single experiment representative of three that gave similar results.

phosphorylation and association of Shc with Flk-1 and the recruitment of Grb2.

In unstimulated cells, the SH3 domains of Grb2 direct the binding to the proline-rich carboxy-terminal region of Sos. Thus, growth factor-induced tyrosine phosphorylation of Shc results in the formation of a Shc/Grb2/Sos complex. Sos is then targeted to the plasma membrane and, through its exchange activity, converts Ras-GDP to active Ras GTP-bound state. Grb2-Sos dissociation occurs through phosphorylation of Sos, as it was reported for other growth factors (34). We demonstrated, as in other cell types (33, 34), that Grb2 and Sos form a complex in BBE cells. VEGF treatment for 5 min caused a partial dissociation (30%) of the complex and a concomitant increase of Sos phosphorylation. The addition of 16K hPRL inhibited the VEGF-induced disruption of the complex and Sos phosphorylation. As for other mitogens, the mechanism underlying this dissociation is still not known. However, it has been reported that MAPK and/or MEK were the phosphorylating kinases involved in this negative feedback mechanism (35, 37, 38). Consistent with this hypothesis, 16K hPRL, which inhibits VEGF-induced activation of MAPK and MEK, could account for the blockade of Grb2/Sos complex dissociation in response to VEGF. Since removal of this feedback

mechanism by 16K hPRL does not result in the maintenance of Ras in a GTP-bound active state, it appears that 16K hPRL is inhibiting Ras activation downstream of the Grb2-Sos complex, in close proximity to Ras. However, the possibility exists that these results reflect the effects of both VEGF and 16K hPRL on total Grb2-Sos complex within the cell and not only on that bound to Flk-1. Since we were unable to detect Sos after Flk-1 immunoprecipitation, we hypothesize that only a small fraction of Grb2-Sos complex was associated with Flk-1, as reported for the epidermal growth factor receptor (39). If that is the case, we expect that the effect of 16K hPRL on the Grb2-Sos complex bound to Flk-1 might not be detected.

Ras has a very low intrinsic GTPase activity, and its inactivation is dependent on Ras-GAP. Ras-GAP functions as a negative regulator by accelerating the conversion of active GTP-bound Ras to the inactive GDP-bound state (Ref. 40; for review, see Ref. 41). In the amino-terminal portion, Ras-GAP contains two SH2 domains flanking one SH3 domain, and the GTPase catalytic activity is within the carboxy-terminal domain. The SH2 domains of Ras-GAP were shown to interact with activated growth factor receptor kinases, e.g. the epidermal growth factor and the platelet-derived growth factor receptors (42). Previous stud-



**Fig. 7.** Signaling Pathways for the Action of 16K hPRL, VEGF, and ISO

All pathways in which 16K hPRL had no effect are shown as *stippled*. Signaling events affected by 16K hPRL are shown in **bold**. Stimulatory events are denoted by a *solid line ending in an arrow* while inhibitory events are shown by a *broken line ending in a vertical line*. Activation of the Flk-1 and its association with Shc/Grb2 and Sos are unaffected by the simultaneous treatment with 16K hPRL. Treatment with 16K hPRL inhibits activation of Ras and the downstream events in the signaling cascade: Raf-1, MEK, and MAPK. The inhibition of Ras activation is correlated with an increased phosphorylation of Ras-GAP; however, the significance of this event on Ras activity is unknown. 16K hPRL, like ISO, stimulates the rate of cAMP formation; however, the action of ISO is mediated via activation of AC but that of 16K hPRL is not. Activation of PKA by increased cAMP levels is known to inhibit Raf-1 activity. The initial signaling events after the occupancy of the 16K hPRL receptor are unknown and await cloning of the receptor. However, these findings suggest that the receptor signals via an undescribed pathway to inhibit Ras activation.

ies have demonstrated that tyrosine 457 (Tyr 457) in bovine Ras-GAP is the major phosphorylation site (43). In addition to Tyr 457, serine and threonine phosphorylation sites were identified, but their localization remains unclear (43). VEGF treatment of BBE cells resulted in an increase in tyrosine phosphorylation of proteins migrating at 120 kDa. Interestingly, the level of phosphorylation of these proteins, comigrating with Ras-GAP, was further increased by addition of 16K hPRL. One hypothesis, consistent with these observations, is that the inhibition of Ras activation by 16K hPRL is mediated through the hyperphosphorylation of Ras-GAP. However, the effect of tyrosine phosphorylation on Ras-GAP activity is unclear (42).

In conclusion, the modulation of VEGF signaling by 16K hPRL appears to be mediated via the inhibition of Ras activation. This study demonstrates, for the first time, the ability of a regulatory factor to inhibit the activation of Ras. Since Ras is generally acknowledged to be a critical event in mitogen-stimulated cell proliferation (27, 36), the antagonism of VEGF-induced Ras activation by 16K hPRL represents a novel and potentially important mechanism for the regulation of angiogenesis. These studies raise a number of interesting questions. One issue is whether the ability of Sos to activate Ras is prevented by a mechanism other than its dissociation from Flk-1-bound Grb2-

Sos complex. Indeed, phosphatidylinositol 4,5-diphosphate has recently been shown to bind and inhibit Sos activity (44). Second, the relationship between the phosphorylation level and the activity of Ras-GAP remains to be clarified. If the phosphorylation of Ras-GAP increases its ability to hydrolyze GTP, this could represent a mechanism for the maintenance of Ras in the inactive state by 16K hPRL. Alternatively, regulation of Ras-GAP activity could occur through formation of a complex with other Ras-GAP-associated proteins such as p62 and p190 (45). Studies are currently being conducted to address these issues.

The initial signaling events in the mediation of the action of 16K hPRL on the MAPK-signaling pathway are still unknown. Although all of the effects on the MAPK cascade are observed only after treatment with VEGF or bFGF (22), direct effects of 16K hPRL have been observed. Treatment of BBE cells with 16K hPRL increases the level of expression of the PAI-1 gene (46) and activates apoptosis, resulting in increased DNA fragmentation (47). We have shown that 16K hPRL activates the caspase cascade, but again the initial signaling events are yet to be discovered. Obviously the signaling events mediating these pleiotropic actions of 16K hPRL will only be understood after the cloning of the receptor.

## MATERIAL AND METHODS

### Production of Recombinant 16K hPRL

For production of intact recombinant 23K hPRL, the coding region of hPRL cDNA minus the signal peptide was inserted into the recombinant plasmid pT7L (48). An ATG was genetically engineered 5' to Leu 1 (49). 16K hPRL-123 was generated by site-directed mutagenesis as previously reported (7). Briefly, cysteine 58 (TGG) of the above construction was mutated to serine (TCC) to prevent the formation of incorrect disulfide bonds, and Lys 124 (AAA) was mutated to a stop codon (TAA). Purification was performed as previously described (7). Purity was greater than 90% and endotoxin levels were below 0.0018 endotoxin units/16 ng.

### Cell Culture

BBE cells were isolated as previously described (50). The cells were grown and serially passaged in low-glucose DMEM supplemented with 10% calf serum (CS), 2 mM L-glutamine, and antibiotics (100 U of penicillin/streptomycin per ml and 2.5 mg of fungizone per ml). Recombinant human bFGF (Promega Corp., Madison, WI) was added (1 ng/ml) to the cultures every other day. Experiments were initiated with confluent cells between passages 5 and 13.

### Cell Stimulation and Preparation of Cell Extracts

Confluent BBE cells were dispersed and plated at a density of  $5 \times 10^5$  cells per 60-mm culture plate (one plate per condition) in DMEM containing 1 ng/ml bFGF. Twenty four hours after plating, cells were serum starved in DMEM containing 0.5% CS for 48 h. Cells were left untreated or treated with 1 nM recombinant human VEGF<sub>165</sub> (N. Ferrara, Genentech, Inc., South San Francisco, CA), 1 nM VEGF plus 1 nM 16K hPRL, or 1 nM 16K hPRL for 5 min at 37°C, to reach a maximal stimulation of MAPK activity as we previously demonstrated (22). For experiments testing the effect of H89, a specific inhibitor of PKA (29), cells were incubated with different concentrations of H89 for 60 min before stimulation with the above factors. Incubations were terminated by aspiration of the medium, two washes with ice-cold PBS, and addition of 200  $\mu$ l of lysis buffer as previously described (22).

### Cell Fractionation

After incubation with the relevant agonists, cells were washed twice with cold PBS, scraped on ice into 500  $\mu$ l of lysis buffer [10 mM Tris-HCl (pH 7.5), 25 mM NaF, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol (DTT), 100  $\mu$ M sodium vanadate, 10  $\mu$ g/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), 2 mM leupeptin, and 0.14 U of aprotinin per ml]. After 15 min on ice to allow swelling, cells were homogenized with 80 strokes in a Dounce homogenizer. Cell nuclei were removed by centrifugation at 4°C for 5 min at  $3,000 \times g$ . Supernatants were centrifuged at 4°C for 30 min at  $100,000 \times g$ . The supernatants (S100 fractions) were removed and stored for further analysis. The pellets were washed twice (centrifugation at 4°C for 15 min at  $100,000 \times g$ ) in 500  $\mu$ l of lysis buffer containing 500 mM NaCl, and once in lysis buffer to remove any MAPK and MEK activity associated with the pellet (27). The washed pellets were resuspended in 200  $\mu$ l of lysis buffer containing 1% NP-40 and incubated on ice for 15 min, and the NP40-soluble fractions (P100 fractions) were stored for further analysis.

### Assay for Raf-1 Activity

Raf-1 was immunoprecipitated from equal quantities of protein from cell lysates and S100- and NP40-soluble fractions

(P100 fractions) from control or stimulated BBE cells. Ten microliters of the specific Raf-1 polyclonal antiserum (Raf-1 (C12), Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to samples for 12–16 h at 4°C, followed by protein A-Sepharose beads for the last 60 min. Immune complexes were washed twice in lysis buffer containing 1% NP-40 and once in kinase buffer [50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 100  $\mu$ M sodium vanadate]. To assay Raf-1 kinase activity in a linked *in vitro* assay, precipitates were resuspended in 10  $\mu$ l kinase buffer and incubated for 20 min at 30°C with 0.5  $\mu$ g of recombinant baculovirus-expressed catalytically active MEK, 1  $\mu$ g of GST-ERK1 [S. Macdonald, Onyx Pharmaceuticals Inc., Richmond, CA; (28,51)], and 10  $\mu$ M ATP. Then 10  $\mu$ l of kinase buffer containing 50  $\mu$ M ATP, 320  $\mu$ g of MBP, and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP were added to each sample and incubated for 10 min at 30°C. Both assays were terminated by the addition of hot 4  $\times$  SDS-PAGE sample buffer followed by boiling for 5 min. Reaction products were resolved by SDS-PAGE (12% gels). Gels were dried and subjected to autoradiography. The radioactivity incorporated into MEK, GST-ERK, and MBP was quantitated by phosphorimaging.

### cAMP Studies

For cAMP studies, cells were washed with 3 ml of fresh medium and then cultured with various concentrations of IBMX (Calbiochem, La Jolla, CA) for 90 min. Cells were challenged with 1 nM 16K hPRL for the indicated times. At the end of the incubation period, the medium was discarded, the cells were lysed in 0.25 ml ice-cold 0.1 N HCl and immediately frozen on dry ice. Subsequently, the cells were sonicated (10 sec), incubated at 4°C for 48 h, and centrifuged ( $2,000 \times g$  for 30 min), and the supernatants were analyzed for cAMP by RIA (52). All samples from an experiment were analyzed in the same assay. The limit of detection was 10 fmol/ml, and the intraassay coefficient of variation was less than 3%.

### AC Assay

Membranes were prepared by nitrogen cavitation as previously described (21, 53). Membranes (0.3 mg/ml) were incubated for 15 min at 30°C in a volume of 50  $\mu$ l containing 50 mM Tris (pH 8), 1 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 2 mM  $\beta$ -mercaptoethanol, 1  $\mu$ g/ml BSA, 10 mM creatine phosphate (Sigma Chemical Co.), 100 U of creatine phosphokinase per ml (Sigma Chemical Co.), 1 mM cAMP (Sigma Chemical Co.); 0.4 mM ATP, 0.1  $\mu$ Ci of [<sup>3</sup>H]cAMP (25–40 Ci/mmol; DuPont NEN, Boston, MA), 4  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]ATP (3,000 Ci/mmol; DuPont NEN), and various activators. Reactions were terminated by addition of 1 ml of 0.5% SDS, and cAMP was isolated as described (54). Under these conditions, the rate of cAMP synthesis was constant during the time of incubation.

### Subcellular Localization of Raf-1

Confluent BBE cell cultures were dispersed and plated at 125,000 cells per chamber slide precoated with 100  $\mu$ g/ml poly-L-lysine in 1 ml of incubation media containing 1 ng/ml bFGF. Twenty four hours after plating, cells were serum starved in DMEM containing 0.5% CS for 48 h. Cells were left untreated or stimulated for 5 min with 1 nM VEGF, 1 nM 16K hPRL, or both factors for 5 min at 37°C. Cells were then fixed for 15 min in ice-cold methanol, washed in PBS, and permeabilized in PBS containing 0.1% saponin for 20 min. After blocking with 5% donkey serum in PBS, cells were incubated with anti-Raf-1 polyclonal antiserum (1:100) for 60 min. After washing, they were incubated with rhodamine-donkey anti-rabbit Ig (1:100) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 5% donkey serum in PBS, washed, and mounted. Immunofluorescence was performed with a Zeiss-Axiophot microscope (Carl Zeiss, Thornwood, NY).

## Determination of GTP-Bound Ras

Confluent BBE cells were dispersed and plated at a density of 500,000 cells per 60-mm culture plate (one plate per condition) in DMEM containing 1 ng/ml bFGF. Twenty four hours after plating, cells were serum starved in DMEM containing 0.5% CS for 36 h and then exposed for 12 h with 0.8 mCi/ml of [<sup>32</sup>P]-orthophosphate (PO<sub>4</sub><sup>2-</sup>) in 3 ml of phosphate-free DMEM. Cells were either left untreated or stimulated with 1 nM VEGF, 1 nM 16K hPRL, or both factors in duplicate for 5 min, and incubations were terminated by washing with ice-cold Tris-buffered saline. Cells were lysed in lysis buffer [0.5% NP-40, 50 mM Tris-HCl (pH 7.5), 20 mM MgCl<sub>2</sub>, 150 mM NaCl, 1% aprotinin, 1 mM Pefabloc] and Ras was immunoprecipitated with anti-Ras monoclonal antibody (Y13-259, 1:10 dilution; Santa Cruz Biotechnology, Inc.) precoupled to protein A-Sepharose by rabbit antirat Ig for 60 min at 4°C (55). Immunoprecipitates were washed three times in lysis buffer and three times in washing buffer [50 mM Tris-HCl (pH 7.5), 20 mM MgCl<sub>2</sub>, 150 mM NaCl]. Ras-associated guanylnucleotides were eluted with 20 mM EDTA, 20 mM DTT, 4% SDS, 0.5 mM GDP, 0.5 mM GTP for 5 min at 68°C. Eluted GDP and GTP were resolved on polyethyleneimine cellulose plates (J.T. Baker, Inc., Phillipsburg, NJ) by TLC using 0.75 M KH<sub>2</sub>PO<sub>4</sub> (pH 3.4) as the solvent. Labeled nucleotides were visualized by autoradiography, and the radioactivity in GTP and GDP was determined by phosphorimaging. Results are as (2/3 × GTP/GDP + 2/3 × GDP) × 100.

## Immunoprecipitation

Total cell lysates (450 µg protein) from control or stimulated BBE cells were immunoprecipitated for 12 h at 4°C with 0.5 µg of antimouse Flk-1 rabbit polyclonal antiserum (gift from Dr. N. Ferrara, Genentech, Inc.); 4 µg of antihuman Grb2-NT rabbit polyclonal antiserum (Upstate Biotechnology, Inc., Lake Placid, NY); or 4 µg of antimouse Sos1 rabbit polyclonal antiserum (Upstate Biotechnology, Inc.). Immune complexes were purified with protein A-Sepharose and washed three times in lysis buffer. Precipitates were subjected to Western blot analysis.

## Western Blot Analysis

P100 and S100 fractions (normalized for protein content) or immunoprecipitates were resolved by SDS-PAGE (6, 7.5, 8, 12%) gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Western blots were probed with the following antibodies: anti-Raf-1 polyclonal antiserum (1:1,000 dilution); anti-Flk-1 rabbit polyclonal antiserum (1:500 dilution); antihuman Shc mouse monoclonal antiserum (1:500 dilution, Santa Cruz Biotechnology, Inc.); antihuman Shc rabbit polyclonal antibody (1:1,000 dilution, Upstate Biotechnology, Inc.); anti-Ash/Grb2 mouse monoclonal antiserum (1:1,000 dilution, Upstate Biotechnology, Inc.); antihuman GAP (Ras-GAP) mouse monoclonal antibody (1:1,000 dilution); antimouse Sos1 rabbit polyclonal antiserum (1:250 dilution); or antiphosphotyrosine mouse monoclonal antiserum (4G10, 1:1,000 dilution, Upstate Biotechnology, Inc.). Western blots were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.05% (vol/vol) Tween 20. Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence reagent (DuPont NEN). The blots were exposed to Reflection NEF films (DuPont NEN). Western blots were "stripped" for reprobing with additional antibodies by incubation for 30 min at 22°C in a buffer containing 0.2 M glycine (pH 2.5) followed by two washes in PBS. Autoradiographs of the different Western blots were analyzed by densitometry and quantified using Intelligent Quantifier (Bio Image, Ann Arbor, MI) software.

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