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Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin

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ABSTRACT A number of factors both stimulating and inhibiting angiogenesis have been described. In the current work, we demonstrate that the angiogenic factor vascular endothelial growth factor (VEGF) activates mitogen-activated protein kinase (MAPK) as has been previously shown for basic fibroblast growth factor. The antiangiogenic factor 16-kDa N-terminal fragment of human prolactin inhibits activation of MAPK distal to autophosphorylation of the putative VEGF receptor, Flk-1, and phospholipase C- γ . These data show that activation and inhibition of MAPK may play a central role in the control of angiogenesis.

The formation of new capillaries from an established microvasculature, angiogenesis, involves stimulation of endothelial cell proliferation, migration, and organization (1, 2). Neovascularization is under the control of angiogenic factors (3, 4), which include members of the fibroblast growth factor family (FGF) (5, 6)—e.g., basic (b) FGF—and the newly discovered vascular endothelial growth factor (VEGF) (7) or vascular permeability factor (8). *In vivo*, both factors have been shown to stimulate the formation of new capillary beds (9). *In vitro*, both factors stimulate cell proliferation of vascular endothelial cells from a variety of sources including bovine brain capillary endothelial (BBE) cells (10, 11). In addition to the angiogenic factors, there appears to be an equally important series of factors that inhibit angiogenesis. Inhibitors of angiogenesis include thrombospondin (12), angiostatin (13), platelet factor 4 (14), and the 16-kDa N-terminal fragment of prolactin (16K PRL) (11). *In vitro* and *in vivo* antiangiogenic factors have been shown to antagonize the proliferation of capillary endothelial cells and neovascularization, respectively (11, 12, 14).

The actions of both bFGF and VEGF are regulated by receptors in the tyrosine kinase family (15, 16). A considerable amount of data already exist on the mechanism of action of bFGF (see ref. 17). The action of VEGF, however, appears to be mediated through two receptors, the human fms-like tyrosine kinase (Flt-1) (16) and Flk-1 (18, 19), the mouse homologue of the human KDR receptor (20). The cytoplasmic domains of these receptors have intrinsic tyrosine kinase activity and sites for autophosphorylation. Occupancy of these receptors results in dimer formation, activation of the tyrosine kinase domains, and autophosphorylation of the receptors (21). Activation of the FGF receptors, in turn, activates a phosphorylation cascade, which regulates the entry of cells into the cell cycle. Phospholipase C- γ (PLC- γ) binds to the activated receptor and is phosphorylated. The ras/raf pathway is also activated, resulting in phosphorylation of the mitogen-activated protein kinases (MAPKs) p42 and p44 and subsequent stimulation of transcription of early response genes (22,

23). In contrast to the well-elucidated mechanism of FGF receptor action, the distal signaling pathway for VEGF receptors has not been determined, although it is known that VEGF rapidly increases the intracellular Ca²⁺ concentration in endothelial cells (24).

To date, the mechanism of action of factors known to inhibit angiogenesis has not been studied. Thus far, it has been shown that 16K PRL inhibits the proliferative action of both bFGF and VEGF on BBE cells (10, 11) by binding to a high-affinity membrane receptor, which appears to be a unique protein (25). However, the structure of the binding site and the pathway by which it signals are unknown.

In this study, we provide evidence that VEGF signals via activation of the MAPK pathway in BBE cells. In addition, 16K PRL inhibits the bFGF- and VEGF-induced phosphorylation and activation of p42 and p44 MAPK. 16K PRL inhibits activation of MAPK by VEGF by acting distal to autophosphorylation of the Flk-1 receptor or phosphorylation of PLC- γ . These findings suggest that activation of MAPK is essential for the proliferative action of bFGF and VEGF and that blockade of this activation mediates the antiangiogenic action of 16K PRL.

MATERIALS AND METHODS

Production of Recombinant Human (h) 16K hPRL. For production of intact 23K hPRL, the coding region of hPRL cDNA minus the signal peptide was inserted into the recombinant plasmid pt7L (26). An ATG was genetically engineered 5' to Leu-1 (27). 16K hPRL was generated by site-directed mutagenesis as reported (11). Briefly, Cys-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 described (11). Purity was >90% and the endotoxin level was <0.0018 unit/16 ng.

Cell Culture. BBE cells were isolated as described (28). The cells were grown and serially passaged in low glucose Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine, and antibiotics (100 units of penicillin/streptomycin per ml and 2.5 μ g of fungizone per ml). bFGF (Promega) was added (1 ng/ml) to the cultures every other day. Experiments were initiated with confluent cells between passages 5 and 13.

Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; VEGF, vascular endothelial growth factor; BBE cell, bovine brain capillary endothelial cell; 16K PRL, 16-kDa N-terminal fragment of prolactin; hPRL, recombinant human PRL; PLC- γ , phospholipase C- γ ; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein.

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Cell Stimulation and Preparation of Cell Extracts. Confluent cell cultures were dispersed and plated at the density of 5×10^5 cells per 60-mm culture plate (one plate per condition) in minimum essential medium (MEM) containing 1 ng of bFGF per ml. Twenty-four hours after plating, cells were serum starved in MEM containing 0.5% calf serum for 24 h. Cells were treated with recombinant human VEGF-165 (VEGF; Genentech), bFGF, and 16K hPRL alone or in combination for different times as indicated. Incubations were terminated by aspiration of the medium and addition of 200 μ l of lysis buffer [1% Triton X-100 lysis buffer containing 20 mM Tris-HCl (pH 8), 137 mM NaCl, 10% (vol/vol) glycerol, 2 mM EDTA, 1 mM Pefabloc, 0.14 unit of aprotinin per ml, 20 μ M leupeptin, and 1 mM sodium orthovanadate] at 4°C for 20 min (29). Insoluble material was removed by centrifugation at 4°C for 10 min at $14,000 \times g$ and the protein concentrations of the soluble fraction were determined with the BCA protein assay kit (Pierce).

Western Blotting Analysis. Cellular proteins were resolved by SDS/PAGE (8%, 12%, 12.5%) and transferred to nitrocellulose membranes (Schleicher & Schuell). Western blots were probed with the following antibodies: an anti-phosphotyrosine mouse monoclonal antibody (4G10; 1:2000 dilution); an anti-Flk-1 rabbit polyclonal antiserum (1:500 dilution) (N. Ferrara, Genentech); an anti-PLC- γ -1 mixed monoclonal antibody (1:1000 dilution); and an anti-MAPK polyclonal antiserum that recognizes both p42 and p44 MAPKs (erk 1-CT; 1:10,000 dilution). Western blots were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.5% Nonidet P-40 and 0.1% Tween 20. Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence system (ECL; Amersham). The blots were exposed to reflection NEF films (NEN). Western blots were "stripped" for reprobing with other primary 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.

In-Gel MAPK Assay. The in-gel MAPK assay was carried out essentially as described (30). Approximately 20 μ g of protein from cell lysates was electrophoresed through a SDS/12.5% polyacrylamide gel containing 0.5 mg of myelin basic protein (MBP) per ml copolymerized in the running gel. After electrophoresis the gel was washed twice in buffer A (50 ml of Tris-HCl, pH 8/5 mM 2-mercaptoethanol) containing 20% isopropyl alcohol and denatured in buffer A containing 6 M guanidine hydrochloride. After the guanidine hydrochloride wash, the proteins in the gel were allowed to renature at 4°C by extensive washing in buffer A containing 0.04% Tween 40. Renatured MBP kinase activity was detected by incubating the gel for 60 min at room temperature in a reaction buffer containing 40 mM Hepes (pH 7.4), 2 mM dithiothreitol (DTT), 15 mM MgCl₂, 300 μ M sodium orthovanadate, 100 mM EGTA, 25 μ M ATP, and 100 μ Ci of [γ -³²P]ATP (1 Ci = 37 GBq). Unincorporated radioactivity was removed by extensive washing in 5% trichloroacetic acid containing 1% tetrasodium pyrophosphate. The gel was dried and exposed to x-ray film for 24 h.

Assay for MAPK Activity. Equal quantities of cell lysates from control or stimulated BBE cells were immunoprecipitated for 12–16 h at 4°C with 1 μ L of MAPK polyclonal antiserum (erk1-CT). Immune complexes were purified with protein A-Sepharose and washed three times in RIPA buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS) and once in kinase buffer (30 mM Tris-HCl, pH 8.0/20 mM MgCl₂/1 mM DTT). The precipitate was resuspended in 30 μ l of kinase assay buffer containing 7 μ g of MBP, 2 μ M ATP, and 5 μ Ci of [γ -³²P]ATP. Samples were incubated for 20 min at 30°C and the assay was terminated by addition of hot 4 \times SDS/PAGE sample buffer (10 ml) 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 the MBP band was quantitated by PhosphorImaging.

RESULTS

Time Course of VEGF-Induced Tyrosine Phosphorylation.

To determine the effect of VEGF on the tyrosine phosphorylation cascade, serum-deprived BBE cells were exposed to 1 nM VEGF for 1–30 min. As shown in Fig. 1, Western blot analysis with a phosphotyrosine antibody revealed four major bands at 200, 160, 44, and 42 kDa. Phosphorylation of the bands was maximal 5 min after treatment with VEGF and declined to undetectable levels by 30 min. Control samples not treated with VEGF showed no changes in tyrosine phosphorylation over the time course of the experiment.

Identification of Phosphorylated Proteins. To confirm the identity of the four phosphorylated proteins after treatment with VEGF (1 nM) for 1, 5, 15, or 30 min, the immunoblot shown in Fig. 1 was stripped and reprobed with specific antibodies to VEGF receptor (Flk-1), PLC- γ -1, or 44- and 42-kDa MAPKs. The 200-kDa tyrosine-phosphorylated band corresponded to the band stained with the antibody to Flk-1 (Fig. 2). A 200-kDa band was also observed in lysates from VEGF-treated cells immunoprecipitated with the antibody to Flk-1 and blotted with the phosphotyrosine antibody. The Flk-1 antibody detected bands of equal intensity at all times after addition of VEGF, demonstrating equal loading and transfer of proteins. The phosphotyrosine bands migrating at 160 kDa corresponded to a band detected with the antibody to PLC- γ -1, while bands at 44 and 42 kDa were stained with the antibody to MAPK.

16K hPRL Inhibits VEGF-Induced Phosphorylation of MAPK. We next asked whether simultaneous treatment with 16K hPRL would inhibit the tyrosine phosphorylation cascade induced by VEGF. Again treatment with 1 nM VEGF for 5 min induced the tyrosine phosphorylation of proteins migrating at 200, 160, 44, and 42 kDa (Fig. 3A, lane 2). Reprobing of the blot with antibodies against specific proteins identified the 160-kDa protein as PLC- γ -1 and the 44- and 42-kDa bands as MAPKs (Fig. 3B and C). These bands were not detected in lysates from control cells (lane 1). Treatment with 1 nM 16K hPRL (lane 4) alone also had no effect on tyrosine phosphorylation of the bands. However, simultaneous treatment with

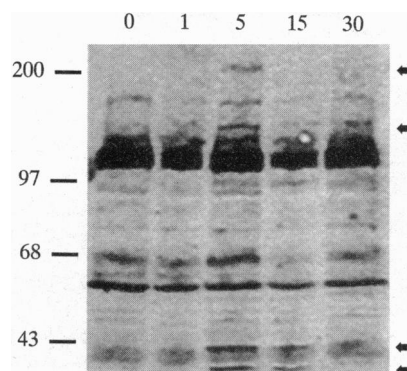


FIG. 1. Time course of tyrosine phosphorylation with ligand-activated VEGF receptor in BBE cells. Quiescent BBE cells were stimulated for 1, 5, 15, or 30 min with 1 nM VEGF or were left untreated before 1% Triton X-100 detergent lysis. Samples were analyzed by SDS/8% PAGE and transferred to a nitrocellulose filter. Tyrosine-phosphorylated proteins were detected by probing the blot with an anti-phosphotyrosine antibody; bands were visualized with an ECL kit (Amersham). Proteins from untreated BBE cell lysates are shown as a control (lane 0). Arrows denote positions of tyrosine-phosphorylated proteins identified with the antibody. Molecular size markers (kDa) are shown on the left.

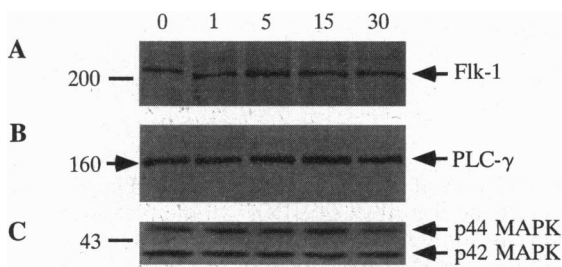


FIG. 2. VEGF induces autophosphorylation of Flk-1 receptor, PLC- γ -1, and MAPK tyrosine phosphorylation. Quiescent BBE cells either untreated (lane 0) or treated with 1 nM VEGF for 1, 5, 15, or 30 min were lysed, and Western blots were prepared as described. Western blots were probed with anti-VEGF receptor (Flk-1) antibody (A), anti-PLC- γ antibody (B), and anti-MAPK antibody (C); bands were visualized with an ECL kit (Amersham). Arrows denote positions of proteins identified with the specific antibodies. Molecular size markers (kDa) are shown on the left.

VEGF and 16K hPRL (lane 3) inhibited VEGF-induced phosphorylation of the 42- and 44-kDa MAPK bands but not the PLC- γ -1 or Flk-1 bands.

16K hPRL Inhibits bFGF-Induced Phosphorylation of MAPK. It was next determined whether 16K hPRL could also block activation of MAPKs by bFGF, another known mitogen for BBE cells. As shown in Fig. 4, the phosphorylated bands corresponding to p42 and p44 MAPKs appear after 5 min of bFGF (0.5 nM) stimulation (lane 2). Cotreatment of cells with 1 nM 16K hPRL prevented the bFGF-induced tyrosine phosphorylation of MAPK (lane 3). Treatment with 16K hPRL alone (lane 4) had no discernable effect over untreated controls (lane 1). Although difficult to observe in Fig. 4, in another experiment 16K hPRL did not inhibit phosphorylation of PLC- γ induced by bFGF treatment (data not shown).

16K hPRL Inhibits VEGF-Induced Activation of MAPK. Immunoblots with the antibody to MAPK of cell lysates resolved on SDS/12.5% polyacrylamide gels showed two major bands of 44 and 42 kDa (Fig. 5). Immunoblots of cells

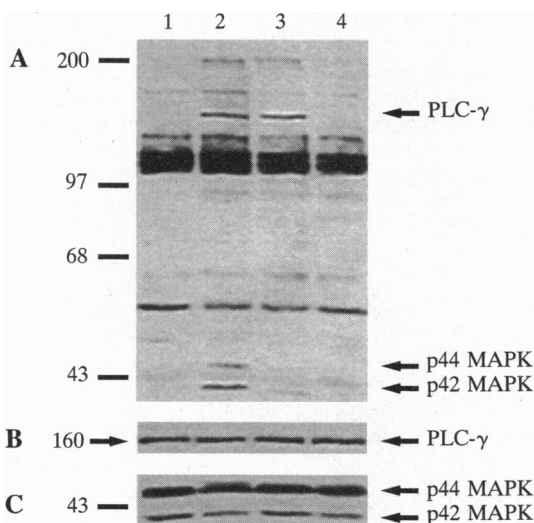


FIG. 3. Inhibition of VEGF-dependent MAPK tyrosine phosphorylation by 16K hPRL. Quiescent BBE cells were left untreated (lane 1) or were stimulated for 5 min with 1 nM VEGF (lane 2), 1 nM 16K hPRL and 1 nM VEGF (lane 3), or 1 nM 16K hPRL (lane 4). (A) Cell extracts were resolved by SDS/8% PAGE, transferred to nitrocellulose filters, and probed with an anti-phosphotyrosine antibody. Positions of proteins of known molecular mass (kDa) and proteins of interest (arrows) are indicated. (B) Immunoblot in A was stripped and re probed with anti-PLC- γ antibody. (C) Immunoblot in B was stripped and re probed with anti-MAPK antibody. B and C show that the proteins were evenly loaded in the gel.

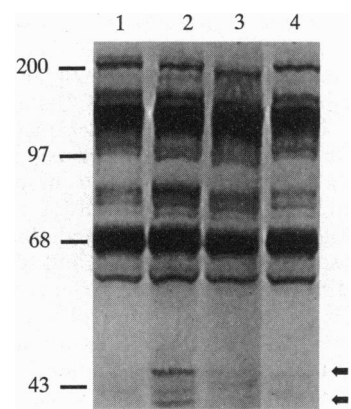


FIG. 4. Inhibition of bFGF-dependent MAPK tyrosine phosphorylation by 16K hPRL. Serum-starved BBE cells were untreated (lane 1) or stimulated for 5 min with 0.5 nM bFGF (lane 2), 1 nM 16K hPRL and 0.5 nM bFGF (lane 3), or 1 nM 16K hPRL (lane 4). Cell extracts were resolved by SDS/8% PAGE, transferred to nitrocellulose filters, and probed with an anti-phosphotyrosine antibody. Positions of proteins of known molecular mass (kDa) and proteins of interest (arrows) are indicated.

treated with 1 nM VEGF for 5 min revealed an apparently higher molecular mass band migrating just above the 44-kDa band (lane 2). In contrast, no molecular mass mobility shift was observed after coincubation of VEGF with 16K hPRL (lane 3). Treatment with 16K hPRL alone had no effect compared to the control (lane 4).

To directly test the effect of 16K hPRL on activation of MAPK by VEGF, experiments were performed using an in-gel enzymatic assay. A 9-fold increase in MAPK activity was observed 5 min after treatment with 1 nM VEGF (Fig. 6A). This VEGF-induced activation was inhibited 70% in the presence of 1 nM 16K hPRL (Fig. 6B). In contrast, 16K hPRL alone had no effect.

The effects of VEGF and 16K hPRL on MAPK activity were also evaluated by the ability of immunoprecipitated MAPK to phosphorylate MBP in solution. As shown in Fig. 7, 5 min after addition of VEGF the activity of MAPK was stimulated nearly 11-fold. In contrast, incubation of cells with 16K hPRL dramatically reduced the stimulatory effect of VEGF on MAPK activity \approx 75%. Again, 16K hPRL alone had little effect on MAPK activity compared with the control.

DISCUSSION

We have previously shown that 16K hPRL inhibits the mitogenic action of bFGF and VEGF on capillary endothelial cells. The goal of the present study was to determine the signaling pathway(s) by which 16K hPRL interfered with the mitogenic action of these angiogenic factors. It was known that both factors signaled through receptors belonging to the tyrosine kinase family and that occupancy of the receptor resulted in autophosphorylation (21). It had also been shown that activation with bFGF initiated a cascade of phosphorylation

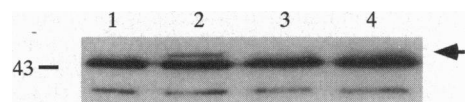


FIG. 5. Inhibition of VEGF-induced molecular mass shift of MAPK by 16K hPRL. Quiescent BBE cells were left untreated (lane 1) or stimulated for 5 min with 1 nM VEGF (lane 2), 1 nM 16K hPRL and 1 nM VEGF (lane 3), or 1 nM 16K hPRL (lane 4). Cell extracts were resolved by SDS/12.5% PAGE, transferred to nitrocellulose filters, and probed with an anti-MAPK antibody. Position of protein of known molecular mass (kDa) and position of protein of interest (arrow) are indicated.

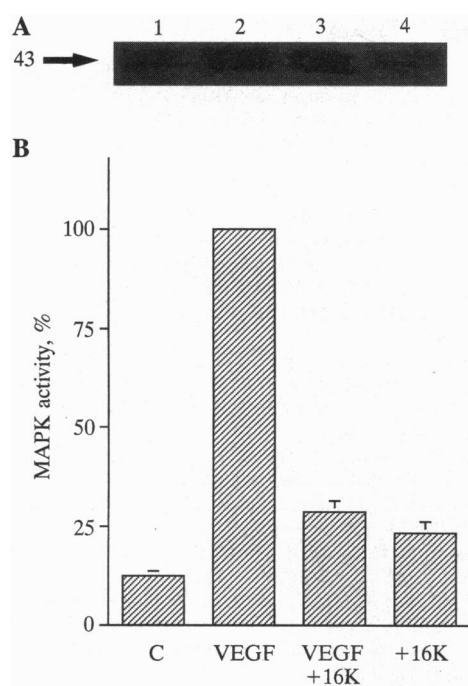


FIG. 6. Inhibition of VEGF-dependent MAPK activity in gel by 16K hPRL. Quiescent BBE cells were left untreated (C, control) or were stimulated for 5 min with 1 nM VEGF, 1 nM VEGF and 1 nM 16K hPRL, or 1 nM 16K hPRL as indicated. (A) Cell lysates were subjected to SDS/PAGE in a gel containing 0.5 mg of MBP per ml. After electrophoresis, the proteins in the gel were successively denatured, renatured, and subjected to an *in situ* kinase reaction with 100 μ Ci of [γ - 32 P]ATP as described. The gel was then dried and autoradiographed. Position of 43-kDa marker is indicated. (B) Radioactivity incorporated into the bands was quantitated by a PhosphorImager. Values represent means of three experiments. Bars indicate SE.

reactions including phosphorylation and activation of MAPK (31). In the current studies we showed, in BBE cells, that in a similar fashion to bFGF, VEGF activates MAPK. We also showed by several criteria that 16K hPRL blocks activation of MAPK induced by bFGF and VEGF. Blockade of the action of VEGF was distal to autophosphorylation of the Flk-1 receptor, a putative receptor for VEGF, and did not appear to involve phosphorylation on PLC- γ .

Recently, VEGF receptors were identified by radioligand binding studies on the membranes of bovine endothelial cells. Cross-linking experiments showed affinity-labeled complexes of 225, 195, and 170 kDa (32–34). The human transmembrane c-fms-like tyrosine kinase (Flt-1) (35) was identified as a receptor for VEGF able to induce Ca^{2+} efflux in *Xenopus* oocytes (16). More recently, VEGF-induced autophosphorylation of Flk-1 was reported in COS-1 cells transiently (18) and in NIH 3T3 cells stably expressing Flk-1 cDNA (19). Porcine aortic endothelial cells transfected with the KDR receptor showed a mitogenic response to VEGF (36). We demonstrated that VEGF rapidly induces the autophosphorylation of Flk-1 in BBE cells. The autophosphorylation of Flk-1 5 min after addition of VEGF is consistent with previous observations in transfected cells expressing Flk-1 cDNA (18, 19).

In addition, VEGF also induced the rapid tyrosine phosphorylation of PLC- γ . Activation of PLC- γ by VEGF is consistent with previous observations. VEGF treatment of cultured endothelial cells resulted in increases in intracellular [Ca^{2+}] and inositol trisphosphate (24). The increased formation of inositol trisphosphate is consistent with activation of PLC- γ and hydrolysis of phosphatidylinositol 4,5-bisphosphate.

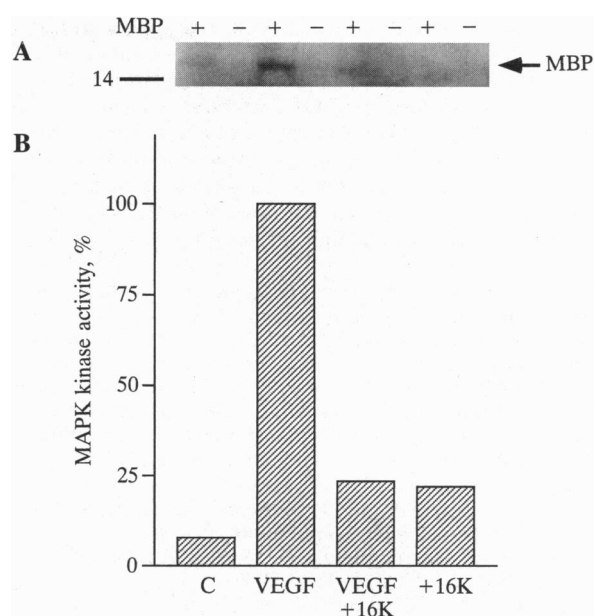


FIG. 7. Inhibition of VEGF-dependent MAPK activity in solution by 16K hPRL. Quiescent BBE cells were left untreated (C, control) or were stimulated for 5 min with 1 nM VEGF, 1 nM VEGF and 1 nM 16K hPRL, or 1 nM 16K hPRL as indicated. (A) Detergent lysates were immunoprecipitated with anti-MAPK antibody, and kinase activity was assayed against MBP in the immune complexes. Reaction mixtures were separated by SDS/PAGE (12% gel) and subjected to autoradiography. Reaction mixtures incubated with MBP (lanes +) and reaction mixtures omitting MBP (lanes -) are indicated. Position of 14-kDa marker is indicated. (B) 32 P in MBP was quantitated by a PhosphorImager. Values shown are averages of two experiments. Data were corrected for background radioactivity in the MAPK immune complexes omitting MBP. Corrected incorporation (VEGF) was set to 100%.

MAPK activation is often observed in cells exposed to mitogens, including bFGF (31). It was shown that the reduced activation of MAPK by antisense expression blocks the proliferative action of bFGF in fibroblasts (37). We have previously demonstrated that both bFGF and VEGF induced proliferation of BBE cells (10, 11). In this study, VEGF was shown to activate MAPK in BBE cells by three criteria: an increase in tyrosine phosphorylation by Western blot analysis with a phosphotyrosine antibody; a characteristic upward shift in electrophoretic mobility of the enzyme; and increased kinase activity both in an in-gel assay and in a solution assay after immunoprecipitation of MAPK. Recently, it was reported that VEGF stimulated phosphorylation of MAPK in rat liver sinusoidal endothelial cells (38). Therefore, it would appear that the VEGF signaling pathway for mitogenesis, like that of bFGF, includes activation of MAPK.

The mitogenic action of both VEGF and bFGF on BBE cells is blocked by 16K hPRL. We now demonstrate that 16K hPRL blocks the phosphorylation and activation of MAPK by VEGF or bFGF. This finding adds additional support to the hypothesis that the mitogenic actions of these angiogenic factors are mediated via activation of MAPK. However, treatment with 16K hPRL did not block VEGF-induced autophosphorylation of the Flk-1 receptor or tyrosine phosphorylation of PLC- γ . The inability of activated PLC- γ , by VEGF or bFGF, to stimulate mitogenesis in the presence of 16K hPRL is consistent with earlier findings that this pathway is not necessary for mediation of the mitogenic action of FGF (39, 40). Mutations in transfected FGF receptors of Tyr766, which blocks activation of PLC- γ , did not block the mitogenic action of bFGF.

The mechanism by which 16K hPRL blocks tyrosine phosphorylation and activation of MAPK is unclear. Our results

suggest that the inhibitory effect occurs at some step distal to autophosphorylation of the receptor and proximal to MAPK. Activation of MAPK after autophosphorylation of several growth factors including bFGF has been shown to involve activation of Ras (31, 41). Activation of Ras triggers a cascade of kinases that result in phosphorylation of both tyrosine and threonine residues of MAPK leading to activation (42). Additional studies are necessary to determine the step at which 16K hPRL blocks the activation of MAPK in this Ras cascade.

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