Tyrosine phosphorylation of the vascular endothelial-growth-factor receptor-2 (VEGFR-2) is modulated by Rho proteins

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The effects of Rho-specific modifying toxins on the tyrosine phosphorylation of endothelial cell proteins were investigated. Incubation of the cells with the Rho-activating toxin cytotoxic necrotizing factor 1 (CNF1) induced a marked increase in the tyrosine phosphorylation of a number of signalling intermediates of the vascular endothelial growth factor (VEGF)-mediated cascade, including focal adhesion kinase, paxillin, phospholipase Cγ1 and a Shc-associated protein of 195 kDa. Both CNF1- and VEGF-dependent tyrosine phosphorylation of these proteins were significantly reduced by prior incubation with C3 transferase, a known inhibitor of RhoA function, suggesting a Rho-dependent mechanism. The stimulation of endothelial cells with CNF1 resulted in a marked increase in the tyrosine phosphorylation of the VEGF receptor (VEGFR)-2, which was correlated with a stimulation of its kinase activity and with its association with downstream tyrosine phosphorylated proteins. The stimulatory effect of CNF1 was specific for VEGFR-2 since the phosphotyrosine content of VEGFR-1 was not affected by the toxin. Transient overexpression of a dominant-active RhoA mutant also induced an increase in the tyrosine phosphorylation of the VEGFR-2, whereas overexpression of a dominant-inactive form of the protein was without effect. Taken together, these results indicate that Rho proteins may play an important role in angiogenesis by modulating the tyrosine phosphorylation levels of VEGFR-2.

Key words: angiogenesis, signal transduction, VEGF.

INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels from pre-existing vessels, is a tightly regulated phenomenon that plays essential roles in reproductive functions, wound healing and embryonic development [1]. However, angiogenesis may become pathological when capillary growth is uncontrolled and the resulting excessive neovascularization may then sustain the development of a number of pathologies including retinopathies, haemangiomas, rheumatoid arthritis, psoriasis and tumour growth [1]. In the latter, it is now clearly established that the aggressive growth of tumours and of their metastases is strictly dependent on angiogenesis [2], suggesting that the inhibition of angiogenesis may represent an effective approach for blocking tumour progression. This hypothesis has been strengthened by the recent demonstration that administration of angiogenesis inhibitors to mice bearing human tumours results in tumour regression [3].

The vascular endothelial growth factor (VEGF) is an endothelial-specific mitogen that is often associated with the activation of tumour-induced angiogenesis. In tumours, VEGF secretion is upregulated by hypoxia [4] and by oncogenes such as *ras* and *src* [5]. At the endothelial cell surface, the secreted protein binds with high affinity to at least two distinct vascular endothelial growth factor receptors (VEGFRs), VEGFR-1, also known as *fms*-like tyrosine kinase (Flt-1) and VEGFR-2, also known as fetal liver kinase (Flk-1) [6]. VEGFR-2 expression in adult endothelial cells appears to account for most of the mitogenic and chemotactic effects of VEGF [7]. At the post-receptor level, activation of endothelial cells by VEGF leads to the autophosphorylation of VEGFR-2 and the subsequent tyrosine phosphorylation of numerous downstream targets, including the second messenger-producing enzymes phospholipase Cγ and phosphoinositide 3-kinase [8,9], p120GAP [8], the adaptor proteins Nck, Grb2 and Shc [8,10], the growth-factor receptor-associated tyrosine phosphatases SHP1 and SHP2 [10], as well as the cytoskeleton-associated proteins p125^{FAK} (where FAK is focal adhesion kinase) and paxillin [11]. The factors involved in the regulation of this VEGF-induced signalling cascade and its overall significance in the numerous biological functions associated with the stimulation of endothelial cells by VEGF, however, remain obscure and additional proteins involved in this signal transduction pathway are likely to exist.

Rho proteins represent a family of monomeric GTPases that play essential roles in all eukaryotic cells studied to date. These proteins act as molecular switches by cycling between active, GTP-bound, and inactive, GDP-bound, states; the cycling between these two states being controlled by a complex network of regulatory proteins [12]. In their active state, Rho proteins interact with a number of downstream targets, leading to the reorganization of the actin cytoskeleton and to cell-cycle progression [13]. The control of these cellular pathways by Rho is of crucial importance for cell adhesion, movement and for a number of morphogenetic processes [12,13].

In the present study, we provide evidence that Rho proteins may also play an important role in other aspects of the signalling pathways triggered by growth factor receptors. We show that in bovine aortic endothelial cells (BAEC), activation of RhoA is both necessary and sufficient to induce the VEGF signalling cascade, possibly by increasing the tyrosine phosphorylation

Abbreviations used: BAEC, bovine aortic endothelial cells; CNF1, cytotoxic necrotinizing factor 1 from *Escherichia coli*; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase; Tyr(P), phosphotyrosine; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; RhoAQ63L, RhoA mutant with glutamine-63 substituted with leucine; PDGF, platelet-derived growth factor.

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levels and kinase activity of the VEGFR-2. These results may have important implications for our understanding of the regulatory mechanisms underlying tumour angiogenesis.

EXPERIMENTAL

Materials

The commercial sources of antibodies used in this study are as follows: agarose-conjugated and unconjugated anti-phosphotyrosine [Tyr(P)] PY20 and anti-paxillin were from Transduction Laboratories; anti-p125^{FAK}, anti-VEGFR-2 (C-terminal), anti-VEGFR-1 and anti-c-Myc (9E10) were from Santa Cruz Biotechnology; anti-phospholipase Cγ and anti-Shc were from Upstate Biotechnology. Human recombinant VEGF was obtained from R&D Systems. The VEGFR-2 tyrosine kinase inhibitor, SU1498, was purchased from Calbiochem.

Cell culture

BAEC were kindly provided by Dr R. Sauvé (University of Montréal, Montreal, Canada). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) bovine fetal serum (HyClone Laboratories), 100 units/ml penicillin, 100 μg/ml streptomycin and were used between passages 17–21. For experimental purposes, cells were plated on Nunc 33-mm 6-well plastic dishes at 10^5 cells/dish and grown to confluence in a humidified atmosphere of 5% CO_2 and 90% air at 37 °C. COS-7 and U-87 cell lines were purchased from the American Tissue Culture Collection and were maintained in minimal essential medium (MEM) containing 10% (v/v) fetal bovine serum.

Purification of recombinant bacterial toxins

The prokaryotic expression vector pGEX-2T containing the cDNA encoding C3 transferase from *Clostridium botulinum* (gift of Dr A. Hall, University College London, London, U.K.) and the pQE-30 vector containing the cDNA encoding the cytotoxic necrotinizing factor (CNF) 1 from *Escherichia coli* (gift of Dr H. Lockman, Uniformed Services University, Bethesda, MD, U.S.A.) were inserted into *E. coli* strain BL21, and fusion proteins were purified from isopropyl-β-D-thiogalactopyranoside-induced exponential-phase bacterial cultures by standard procedures. For C3 transferase, the glutathione *S*-transferase moiety of the fusion protein was removed by incubating the glutathione–Sepharose beads (1 ml) overnight at room temperature with 50 units of thrombin protease (Pharmacia). Contaminating thrombin that was eluted with the recombinant C3 protein was removed by a 1-h incubation of the eluate with 5 μl of *p*-aminobenzamidine-linked agarose beads (Sigma). CNF1 was purified to apparent homogeneity by affinity chromatography on Ni-NTA–Sepharose beads (Qiagen).

VEGF stimulation of BAEC

BAEC grown to confluence were rendered quiescent by a 24-h incubation in serum-free DMEM. The cultures were washed twice with the same medium and incubated for 1 min at 37 °C in 2 ml of serum-free DMEM containing 1 nM of human recombinant VEGF (R&D Systems). After VEGF treatment, cells were washed once with PBS containing 1 mM vanadate and incubated in the same medium for 1 h at 4 °C. The cells were solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 1% (v/v) Triton X-100] containing 1 mM vanadate. The cells were then scraped from the culture dishes and the resulting lysates were clarified by centrifugation at 10000 ***g*** for 10 min. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

Toxin treatments

For incubation of the cells with CNF1, confluent BAEC cultures were washed twice with serum-free DMEM and incubated in 2 ml of DMEM containing 1 μg/ml of purified CNF1. The cells were incubated at 37 °C for 6 h in the presence of the toxin and subsequently solubilized in lysis buffer as described above. To study the effect of SU1498 on the CNF1-induced tyrosine phosphorylation, the inhibitor was added to the cells 1 h before the toxin. For pretreatment of the cells with C3 transferase, confluent cells were washed twice with serum-free DMEM and incubated for 24 h in 2 ml of the same medium containing 50 μg/ml of purified C3 transferase. The medium was aspirated and the cells were processed for VEGF stimulation as described above. The efficiency of ADP-ribosylation of RhoA by C3 transferase in these conditions was determined by monitoring the *in vitro* C3 transferase-catalysed [^{32}P]NAD incorporation into the protein, as described previously [14]. Briefly, lysates (15 μg) were incubated in a mixture containing 100 mM Tris/HCl, pH 8.0, 10 mM thymidine, 5 mM $MgCl_2$, 10 μM [^{32}P]NAD (2 μCi) (Dupont NEN) and 5 μg/ml of C3 transferase. The mixture was incubated at 30 °C for 1 h and the reaction was terminated by the addition of 5-fold concentrated Laemmli sample buffer. The samples were subjected to SDS/PAGE on a 12.5% polyacrylamide gel and the incorporated radioactivity was revealed by autoradiography. The efficiency of the CNF1-catalysed deamidation of RhoA was estimated by the observation of a slight shift in its electrophoretic migration on a 15% polyacrylamide gel [15].

Immunoprecipitation and immunoblotting procedures

For the immunoprecipitation studies, similar amounts of proteins from each sample (usually 50 μg) were clarified by a 1 h incubation at 4 °C with a mixture of Protein A/Protein G–Sepharose beads. Following the removal of the Sepharose beads by low-speed centrifugation, the supernatants were transferred to fresh tubes and incubated in lysis buffer overnight at 4 °C in the presence of 1–4 μg/ml of specific antibodies. With the exception of the agarose-conjugated anti-Tyr(P) monoclonal antibody, the immune complexes were collected by incubating the mixtures with 25 μl (50% suspension) of either Protein A–Sepharose beads (for rabbit polyclonal antibodies) or Protein G–Sepharose beads (for mouse IgG_1 monoclonal antibodies). Non-specific bound proteins were removed by washing the beads three times in 1 ml of lysis buffer, and bound material was solubilized in 25 μl of 2-fold concentrated Laemmli sample buffer, boiled for 4 min and analysed by Western blotting, as described below.

Samples were separated by SDS/PAGE (7.5% gel) and electroblotted on to PVDF membranes using standard procedures. The membranes were blocked overnight in Tris-buffered saline (147 mM NaCl, 20 mM Tris/HCl, pH 7.5) containing 2% (w/v) BSA and probed with primary antibodies for 2 h at room temperature. Immunoreactive bands were visualized after a 1-h incubation with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch Laboratories), followed by enhanced chemiluminescence (Amersham). In some cases, the immunoreactive bands were quantified by scanning densitometry (Molecular Dynamics).

Immune complex kinase assay *in vitro*

Precleared cell lysates (100 μg) were incubated overnight with anti-VEGFR-2 antibodies and the resulting immune complexes were collected using Protein A–Sepharose beads as described above. The beads were washed three times with lysis buffer, followed by two additional washes with kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 1 mM dithiothreitol). The beads were then incubated for 5 min at room temperature in 25 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (ICN Biochemicals) and the reactions were stopped by the addition of 5-fold concentrated Laemmli sample buffer. After electrophoresis on 7.5 % polyacrylamide gels, the gels were incubated for 30 min in 2.5 % glutaraldehyde and incubated at 55 °C for 1 h in 1 M KOH to remove serine-bound phosphate. The resulting gels were washed extensively in methanol/acetic acid (4:1, v/v) and exposed to Fuji films.

Overexpression of RhoA mutants

BAEC (70 % confluent) were transfected with 1 μg of insertless vector (pcDNA3), pcDNA3–c-Myc–RhoAQ63L (dominant-active RhoA mutant where Gln-63 is substituted with Leu) or pcDNA3–c-Myc–RhoAQ63LC190R (dominant-active but non-isoprenylable RhoA mutant) (gifts from W. Moolenaar) using the Fugene6 transfection reagent (Roche Molecular Biochemicals). After 24 h of transfection, cells were lysed as described above and the Tyr(P) content of the VEGFR was determined by immunoprecipitation with anti-VEGFR-2 antibodies followed by immunodetection with the anti-Tyr(P) monoclonal antibody. The levels of overexpressed RhoA mutants in the transfected cells were determined in parallel experiments by immunoblotting the cell lysates with an anti-Myc epitope monoclonal antibody (9E10).

RESULTS

Rho-modifying toxins modulate tyrosine phosphorylation of endothelial cell proteins

VEGF is an endothelial-cell-specific mitogen which is known to induce an increase in the tyrosine phosphorylation of a number of endothelial cell proteins [7–11]. Under our experimental conditions, the addition of VEGF (1 nM) to quiescent BAEC promoted a rapid but transient increase in the tyrosine phosphorylation of a number of endothelial cell proteins, as detected by immunoblotting of anti-Tyr(P) immunoprecipitates (Figure 1A). The tyrosine phosphorylation was maximal at 1 min and rapidly returned to basal levels at longer incubation times. The induction of tyrosine phosphorylation by VEGF, measured under these conditions, was highly reproducible but the extent of stimulation was variable, ranging from 2- to 5-fold.

The effect of the Rho-specific activating toxin, CNF1, on the pattern of tyrosine phosphorylation of these endothelial cell proteins was examined. Quiescent cultures of BAEC were incubated with 1 μg/ml of the recombinant protein for various periods of time and the resulting increase in the amount of tyrosine phosphorylated proteins was monitored, as described in the Experimental section. As shown in Figure 1(B), incubation of the cells in the presence of CNF1 markedly stimulated the tyrosine phosphorylation of cellular proteins in a time-dependent manner. The stimulatory action of CNF1 was preceded by a 2 h-lag period, possibly reflecting its uptake and activation by the endothelial cells [16], and was maximal at 4–8 h and progressively declined at longer incubation times. A similar bell-shaped activation of the tyrosine phosphorylation of fibroblast proteins by CNF1 has been reported [16], and possibly represents an intrinsic feature of the toxin. This CNF1-induced tyrosine phosphorylation was correlated with a decrease in the electrophoretic mobility of RhoA, but not of Cdc42, suggesting the specific modification of RhoA by the toxin (Figure 1C) [15]. Moreover, the endogenous amount of Rho was not altered by CNF1, eliminating the possibility that the stimulatory effect of the toxin was related to an upregulation of RhoA in the cells. Interestingly, this stimulatory effect of CNF1 was several fold higher in endothelial cells than in other cell types. For example, incubation of either COS-7 or glioblastoma U87 cells with CNF1 under conditions resulting in maximal phosphorylation of endothelial cell proteins (6 h) had negligeable effect on the tyrosine phosphorylation of cellular proteins (Figure 1D). Overall, these results suggest that CNF1 is a very potent and specific inducer of tyrosine phosphorylation of endothelial cell proteins.

We next examined whether the inhibition of Rho by C3 transferase could affect the stimulation of tyrosine phosphorylation triggered by VEGF and CNF1. By contrast with CNF1, C3 does not readily enter into cells [17], and we first determined the C3 concentration that would result in a significant modification of endogenous Rho proteins. Quiescent BAEC were starved for 24 h in the presence of increasing concentrations of C3 transferase (0–50 μg/ml) and the resulting cell lysates were subjected to ADP-ribosylation *in vitro* using [^{32}P]NAD as the ribose donor. As shown in Figure 2(A), lysates from cells cultured in the presence of the toxin showed a concentration-dependent decrease in the level of [^{32}P]-labelled Rho (80 % inhibition at 50 μg/ml), indicating prior endogenous ADP-ribosylation.

Incubation of the cells with C3 transferase had a moderate effect on the basal Tyr(P) content of endothelial cell proteins, with the notable exception of a 68-kDa protein, the phosphorylation of which was sensitive to the toxin (Figure 2B). The addition of VEGF or CNF1 to BAEC promoted an increase (2-fold) in the tyrosine phosphorylation of endothelial cell proteins but their stimulatory effects were significantly reduced by prior incubation of the cells with C3 transferase (Figure 2B). Densitometric scanning of a number of gels from different cell preparations indicated that the inhibitory effect of C3 on both CNF1- and VEGF-induced tyrosine phosphorylation was 60 ± 9 % ($n = 8$). These results suggest that the stimulation of tyrosine phosphorylation of endothelial cell proteins by VEGF and CNF1 involves Rho proteins.

CNF1 stimulates the tyrosine phosphorylation of signalling intermediates

VEGF has been shown to stimulate the tyrosine phosphorylation of a number of signalling intermediates [7–11]. To determine whether the substrates phosphorylated, following stimulation of the cells with VEGF, were the same as those in which phosphorylation was increased by CNF1 or decreased following C3 treatment, lysates from cells stimulated with VEGF or from cells preincubated in the presence of the toxins were immunoprecipitated with antibodies directed against proteins that were previously reported to be tyrosine phosphorylated upon stimulation of endothelial cells with VEGF. Using this procedure, p125^{FAK}, paxillin, phospholipase Cγ and a Shc-associated protein of 195 kDa were identified as major substrates phosphorylated in response to VEGF (Figure 3, left and middle panels). Pretreatment of the cells with C3 abolished the VEGF-induced increase of tyrosine phosphorylation of these proteins, with the notable exception of phospholipase Cγ, in which tyrosine phosphorylation was less sensitive to C3 treatment. Interestingly, incubation of the cells with CNF1 stimulated the tyrosine

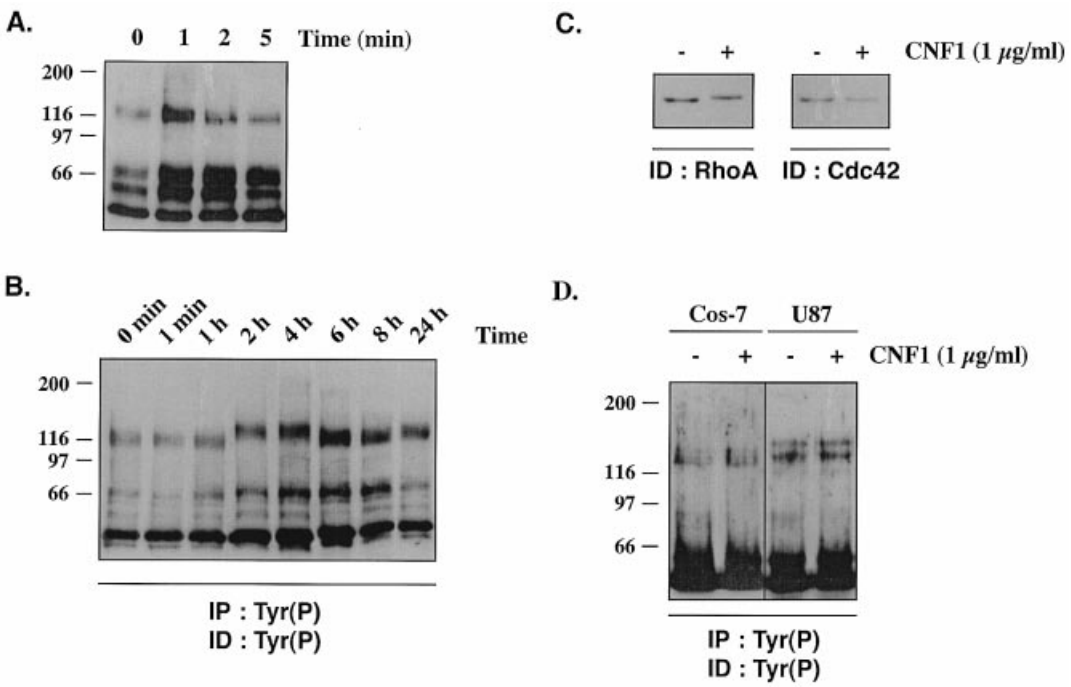

Figure 1 VEGF and CNF1 induce tyrosine phosphorylation of endothelial cell proteins

(**A**) Time course of VEGF-induced tyrosine phosphorylation. Serum-starved quiescent BAEC were incubated in serum-free medium in the presence of 1 nM of recombinant VEGF for the indicated times, and the extent of tyrosine phosphorylation was determined by immunoprecipitation with anti-Tyr(P) followed by immunoblotting with the same antibody. The results are representative of five experiments performed on different cell preparations. Molecular-mass markers (kDa) are shown on the left. (**B**) Effect of CNF1 on the tyrosine phosphorylation of BAEC proteins. Quiescent BAEC were incubated in serum-free medium with 1 μg/ml of recombinant CNF1 for the indicated times. Tyrosine phosphorylated proteins were identified by immunoprecipitation and immunoblotting with the anti-Tyr(P) monoclonal antibody. The results are representative of three experiments performed on different cell preparations. Molecular-mass markers (kDa) are shown on the left. (**C**) Modification of endogenous RhoA by CNF1. Lysate proteins from control cells and from cells incubated for 6 h in the presence of 1 μg/ml of CNF1 were separated on 12.5% polyacrylamide gels and electroblotted on to PVDF membranes. The resulting membranes were probed with anti-RhoA or anti-Cdc42 antibodies. The results are representative of two experiments performed on different cell preparations. (**D**) Specificity of CNF1 action. COS-7 and U87 glioblastoma cells were serum-starved and incubated for 6 h with 1 μg/ml of CNF1. The extent of tyrosine phosphorylation was monitored as described above. The results are representative of two experiments performed on different cell preparations. Molecular-mass markers (kDa) are shown on the left. ID, immunodetection; IP, immunoprecipitation.

phosphorylation of the same proteins (Figure 3, right and middle panels). These results strongly support the notion that Rho proteins play a major role in the tyrosine phosphorylation events triggered by VEGF.

Involvement of VEGFR-2 in CNF1-induced tyrosine phosphorylation

It is now clearly established that the mitogenic effects of VEGF on endothelial cells are related to its specific binding to the Flk receptor tyrosine kinase, VEGFR-2 [6,7,18]. By analogy with other growth factor receptors with intrinsic tyrosine kinase activities, stimulation of VEGFR-2 induces intramolecular tyrosine autophosphorylation of the receptor and subsequent association of the receptor with downstream signalling proteins [6,7,18]. We thus examined if the Rho-specific toxins could exert their effect by directly modulating the Tyr(P) content of the VEGFR-2. Lysates from treated BAEC were immunoprecipitated with anti-VEGFR-2 antibodies and the tyrosine phosphorylation of the receptor and its associated tyrosine phosphorylated proteins was examined by immunoblotting with anti-Tyr(P) antibodies. As expected, stimulation of the cells with VEGF markedly increased the amount of Tyr(P) associated with the 205-kDa VEGFR-2 protein as well as that of several tyrosine phosphorylated proteins that co-immunoprecipitated with the receptor (Figure 4A, left panel). This VEGF-dependent tyrosine phosphorylation of the receptor and of its associated proteins was, however, significantly decreased by preincubation of the cells with C3 transferase prior to stimulation with VEGF. By contrast, immunoprecipitation of VEGFR-2 from cells incubated in the presence of CNF1 showed that the toxin promoted a major increase in the Tyr(P) content of the receptor as well as its association with a number of tyrosine phosphorylated proteins (Figure 4A, left panel). The pattern of tyrosine phosphorylated proteins associated with VEGFR-2 immunoprecipitated from VEGF- and CNF1-stimulated cells was strikingly similar, with the notable exception of a 130-kDa phosphoprotein that appeared to be preferentially associated with VEGFR-2 in CNF1-treated cells.

To determine whether the increase in the phosphorylation of VEGFR-2 induced by CNF1 resulted in a stimulation of its kinase activity, anti-VEGFR-2 immunoprecipitates from control and treated cells were subjected to *in vitro* kinase assays. As shown in Figure 4(A) (right panel), immunoprecipitates from both CNF1- and VEGF-stimulated cells showed a marked increase in the radiolabelling of VEGFR-2 and its associated proteins, indicating that both treatments increased the kinase activity of the receptor. The increase in kinase activity of VEGFR-2 induced by VEGF was however significantly less in immunoprecipitates from C3-treated cells.

We next compared the stimulatory effect of CNF1 on the Tyr(P) content of VEGFR-2 with that of another VEGF receptor,

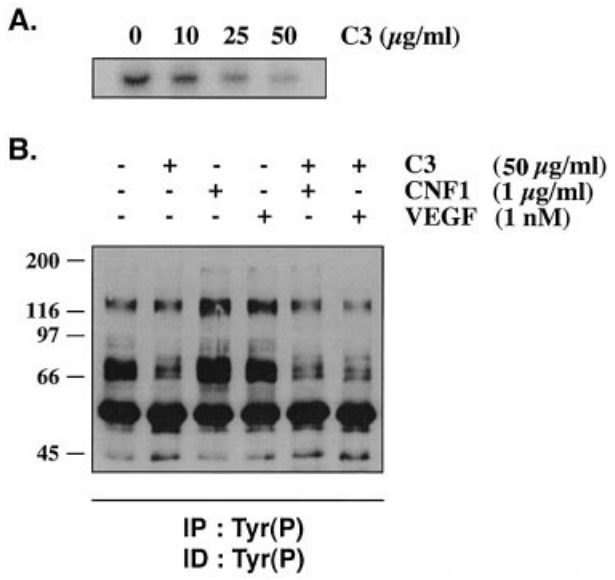

Figure 2 Effect of C3 transferase on the VEGF- and CNF1-induced tyrosine phosphorylation of BAEC proteins

(**A**) ADP-ribosylation of Rho. BAEC were incubated for 24 h in serum-free medium in the absence or in the presence of the indicated concentrations of C3 transferase (C3). The resulting cell lysates were ADP-ribosylated *in vitro*, separated by SDS/PAGE and the incorporated radioactivity was revealed by autoradiography. (**B**) BAEC were serum-starved in the absence or in the presence of 50 μg/ml of purified C3 transferase, followed by incubation of the cells with 1 nM VEGF or 1 μg/ml of CNF1. The resulting tyrosine phosphorylation of endothelial cell proteins was determined as described in the legend to Figure 1. The results are representative of five experiments performed on different cell preparations. ID, immunodetection; IP, immunoprecipitation.

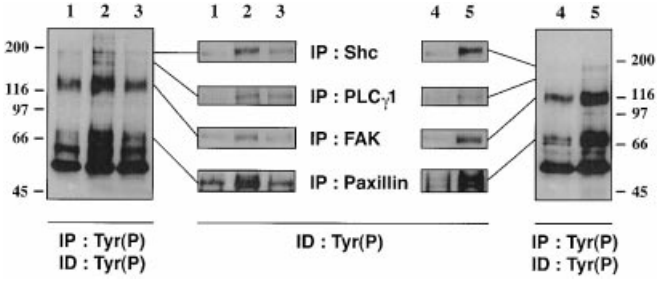

Figure 3 Identification of tyrosine-phosphorylated proteins following treatment of BAEC with VEGF and CNF1

Quiescent BAEC were serum-starved for 24 h in the absence (lanes 1 and 2) or in the presence of C3 transferase (lane 3), followed by a 1-min incubation in the absence (lane 1) or in the presence of 1 nM of VEGF (lanes 2 and 3). In parallel experiments, BAEC were serum-starved for 6 h in the absence (lane 4) or in the presence (lane 5) of 1 μg/ml of CNF1. The corresponding cell lysates were subjected to immunoprecipitation (IP) with agarose-conjugated anti-Tyr(P) (left and right panels) or with the indicated antibodies (middle panel). The immune complexes were resolved by SDS/PAGE and immunodetected with the anti-Tyr(P) monoclonal antibody. The results are representative of two experiments performed on different cell preparations. ID, immunodetection; IP, immunoprecipitation; PLC, phospholipase C.

VEGFR-1. Lysates from CNF1- and VEGF-stimulated cells were subjected to immunoprecipitation with anti-VEGFR-2 or anti-VEGFR-1 antibodies and the Tyr(P) content of the receptors was revealed by immunoblotting with the anti-Tyr(P) antibody. As expected, VEGF promoted a marked increase in the tyrosine phosphorylation of both receptors, with 7- and 2.5-fold increases

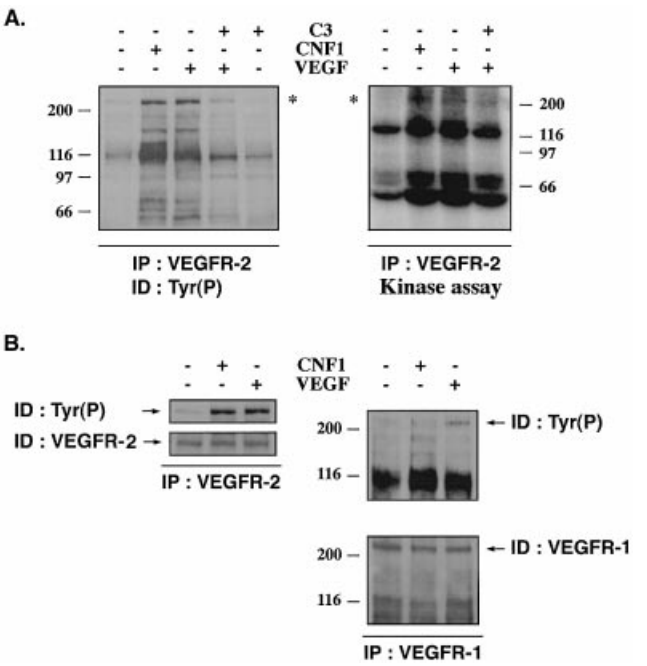

Figure 4 Effect of Rho-specific toxins on the Tyr(P) content of the VEGFR-2 and its associated proteins

(**A**) Left panel: BAEC were incubated with the Rho-specific toxins and VEGF as described in the legends to Figures 1 and 2. The resulting lysates were incubated with polyclonal antibodies raised against the VEGFR-2 and the immune complexes were subjected to SDS/PAGE, followed by immunoblotting with anti-Tyr(P) monoclonal antibody. The results are representative of three experiments performed on different cell preparations. Right panel: anti-VEGFR-2 immunoprecipitates (IP) from control and treated cells were subjected to an *in vitro* kinase assay. Samples were separated by SDS/PAGE, followed by treatment with KOH and autoradiography. The position of VEGFR-2 is indicated by asterisks. The results are representative of two experiments performed on different cell preparations. (**B**) Lysates from control and VEGF- and CNF1-treated cells were immunoprecipitated (IP) with antibodies against (ID) VEGFR-2 (upper panels) or VEGFR-1 (lower panel). The Tyr(P) content of the resulting immune complexes was monitored using the anti-Tyr(P) antibody. The results are representative of two experiments performed on different cell preparations. ID, immunodetection; IP, immunoprecipitation.

for VEGFR-2 and VEGFR-1 respectively (Figure 4B), in agreement with the higher stimulatory effect of VEGF on VEGFR-2 activity reported previously [7]. Interestingly, CNF1 also induced a 7-fold stimulation of the tyrosine phosphorylation of VEGFR-2, whereas it failed to significantly increase the Tyr(P) content of VEGFR-1 (1.1-fold stimulation) and of its associated proteins (Figure 4B, lower panel). The amounts of VEGFR-1 and VEGFR-2 in the immunoprecipitates were constant in all conditions, eliminating the possibility that changes in receptor levels could account for the observed effects. These data thus indicate that modulation of Rho activity by CNF1 triggers a specific modification of the Tyr(P) content of the VEGFR-2 protein that is correlated with an increase in its activity and with its association with downstream tyrosine phosphorylated proteins.

The participation of VEGFR-2 in the increase of tyrosine phosphorylation induced by CNF1 was further investigated by monitoring the effect of a rather specific VEGFR-2 kinase inhibitor, SU1498 [19], on the tyrosine phosphorylation of endothelial cell proteins. Cells were pretreated for 1 h in the presence of low concentrations of SU1498, followed by a 6-h incubation with CNF1. The resulting tyrosine phosphorylation of endothelial cell proteins was monitored by immuno-

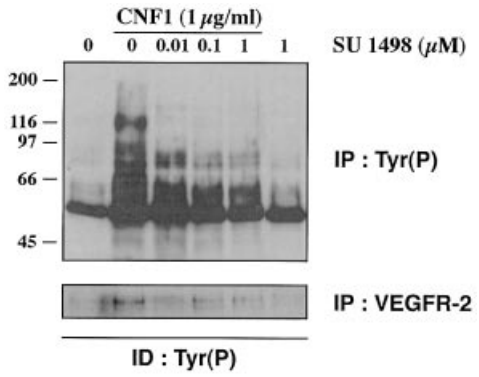

Figure 5 Inhibition of the CNF1-induced tyrosine phosphorylation by the VEGFR-2 inhibitor, SU1498

Quiescent BAEC were preincubated in serum-free medium for 1 h in the presence of vehicle (0) or of increasing concentrations of SU1498. The cells were subsequently incubated for 6 h with 1 μg/ml of CNF1 and the extent of tyrosine phosphorylation was determined following immunoprecipitation (IP) of the cell lysates with anti-Tyr(P) (upper panel) or anti-VEGFR-2 antibodies (lower panel). The results are representative of three experiments performed on different cell preparations. ID, immunodetection; IP, immunoprecipitation.

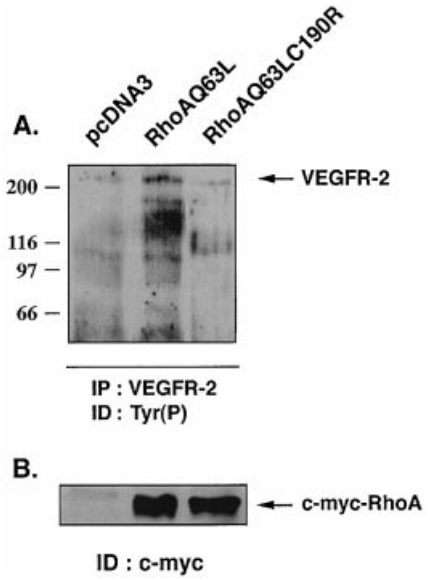

Figure 6 Effect of overexpressed RhoA mutants on the tyrosine phosphorylation of VEGFR-2

(**A**) Subconfluent BAEC were transfected with vector alone (pcDNA3) or vectors encoding the RhoAQ63L and RhoAQ63LC190R mutants. Following transfection, cells were lysed and the Tyr(P) content of the VEGFR-2 was monitored by immunoprecipitation (IP) with anti-VEGFR-2 polyclonal antibodies followed by immunoblotting (ID) with an anti-Tyr(P) monoclonal antibody. The results are representative of two experiments performed on different cell preparations. (**B**) Lysates from transfected cells (10 μg) were separated on 12% polyacrylamide gels and the levels of overexpressed RhoA mutants were determined by immunoblotting (ID) with a monoclonal antibody raised against the c-Myc epitope tag (9E10). ID, immunodetection; IP, immunoprecipitation.

precipitation with the anti-Tyr(P) monoclonal antibody and with the anti-VEGFR-2 antibodies, as described above. As shown in Figure 5 (upper panel), preincubation of the cells with SU1498 resulted in a dose-dependent inhibition of the CNF1-induced tyrosine phosphorylation of BAEC proteins, but had no significant effect on the basal Tyr(P) content of the proteins, up to 1 μM. In these conditions, half-maximal inhibition of the CNF1-induced tyrosine phosphorylation was achieved at a concentration of approx. 0.1 μM, similar to that reported for the inhibition of the VEGF-dependent autophosphorylation of VEGFR-2 [19]. Moreover, the tyrosine phosphorylation of VEGFR-2 induced by CNF1 was inhibited to the same extent by this concentration of SU1498 (Figure 5, lower panel). These results thus strongly support the notion that the CNF1-induced stimulation of the tyrosine phosphorylation of endothelial cell proteins is related to an increase in the VEGFR-2 kinase activity.

The CNF1 toxin has been reported to act as an activator of RhoA, abolishing its GTPase activity by selective deamidation of glutamine-63 of the protein [15,20,21]. This residue is pivotal for Rho deactivation, since it is implicated in the binding of a water molecule required for hydrolysis of the GTP γ-phosphate, a process that is considerably accelerated by rhoGAP [22]. We thus examined whether overexpression of a dominant-active RhoA mutant (RhoAQ63L) could also affect the phosphorylation status of VEGFR-2. As shown in Figure 6(A), BAEC overexpressing RhoAQ63L showed an increase in tyrosine phosphorylation of the VEGFR when compared with cells transfected with the vector alone. This stimulatory effect of the RhoA mutant was much lower than that achieved using CNF1, possibly reflecting the low efficiency of gene transfection in endothelial cells [23]. Nevertheless, RhoAQ63L induced a 2-fold increase in the phosphorylation of the receptor and its association with phosphorylated proteins of 195 and 125 kDa (Figure 6A). Interestingly, the stimulatory effect of the overexpressed protein on the VEGFR-2 Tyr(P) content was abolished in cells overexpressing comparable levels of a non-isoprenylable homologue of RhoAQ63L, RhoAQ63LC190R. These results thus indicate that the stimulatory effect of CNF1 most likely occurs through its activation of RhoA GTPase, and that effective stimulation of the VEGFR-2 tyrosine phosphorylation requires the membrane localization of the protein.

DISCUSSION

The use of bacterial toxins that specifically target Rho proteins has been of crucial importance to our understanding of the key regulatory roles played by these proteins in cellular processes [24]. The recently identified CNF1 toxin is of particular interest since it specifically activates RhoA by the selective deamidation of glutamine-63 (RhoQ63) in the switch 2 domain of the protein [15,20,21]. This modification of RhoQ63 to RhoE63 by CNF1 blocks the intrinsic and GAP-stimulated hydrolysis of GTP [22], and thus results in the constitutive activation of Rho [20,21]. In addition, CNF1 readily enters cultured cells and, following its activation in the cytosol, induces changes in cell architecture that are indistinguishable from those observed following overexpression of the constitutively active form of this protein [15,17,20,21].

In the present work, we observed that the incubation of cultured BAEC with CNF1 markedly increased the tyrosine phosphorylation of a wide variety of endothelial cell proteins. An increase in the tyrosine phosphorylation of $p125^{FAK}$ and paxillin following treatment of fibroblasts and Chinese-hamster ovary cells with CNF1 has been reported [16,25], but to our knowledge our results using BAEC as a model represent the first evidence that CNF1 can stimulate the tyrosine phosphorylation of other key intermediates of growth factor-induced signalling cascades.

This CNF1-dependent increase in tyrosine phosphorylation is mediated by Rho proteins, since it was correlated with the

modification of endogenous RhoA and was completely abolished by prior incubation of the cells with C3 transferase, a well-characterized inhibitor of Rho function. Moreover, immunoprecipitation experiments indicated that CNF1 stimulated the tyrosine phosphorylation of substrates of the VEGF-mediated signalling cascade, such as phospholipase Cγ, p125^{FAK}, paxillin and a previously unidentified 195-kDa Shc-associated protein. The observation that CNF1 induced an increase in the tyrosine phosphorylation of these proteins, and C3 inhibited the VEGF-dependent phosphorylation of these substrates, strongly suggests that Rho proteins play a role in the endothelial cell response to VEGF.

We were interested to determine whether the increase in tyrosine phosphorylation induced by CNF1 could be related to activation of VEGFR-2 kinase activity, since it is well-established that autophosphorylation of VEGFR-2 on tyrosine residues represents the initial step of the VEGF-induced signalling cascade [7,18]. In this respect, we observed that the stimulation of BAEC with VEGF resulted in a marked increase in the tyrosine phosphorylation of VEGFR-2 and of a number of receptor-associated phosphorylated proteins, whereas phosphorylation of VEGFR-1 was stimulated to a much lesser extent by VEGF. Perhaps surprisingly, CNF1 induced an increase in the Tyr(P) content of VEGFR-2 and of its associated tyrosine phosphorylated proteins, which showed a striking similarity to that induced by VEGF, but pretreatment of the cells with C3 transferase inhibited the VEGF-dependent tyrosine phosphorylation of VEGFR-2. Furthermore, this increase in VEGFR-2 Tyr(P) content was correlated with an increase in kinase activity towards its associated proteins. The stimulatory effect of CNF1 on VEGFR phosphorylation was specific for VEGFR-2, as treatment of the cells with the toxin failed to increase the VEGFR-1 Tyr(P) content. The involvement of Rho proteins in the regulation of the VEGFR-2 tyrosine phosphorylation levels was also strengthened by the observation that the overexpression of a dominant-active RhoA mutant (RhoAQ63L) resulted in a similar increase in the VEGFR-2 tyrosine phosphorylation. Taken together, these results suggest that Rho proteins may represent a key intermediate in the signal transduction pathways involved in the response of endothelial cells to VEGF by modulating the activity of the most upstream component of this pathway.

Although receptor tyrosine kinase phosphorylation has been associated with ligand-induced dimerization and auto- or trans-phosphorylation of the receptors [26], other evidence has pointed to the existence of an equilibrium between monomeric and dimeric receptors in the absence of ligand [27]. In the case of VEGFR-2, monomeric forms of the extracellular domain of the receptor bind VEGF 100 times more weakly than its dimeric forms, suggesting a component with strong avidity for binding VEGF to predimerized forms of the receptor [28]. Moreover, NIH 3T3 cells overexpressing VEGFR-2 showed a higher proliferation rate in the absence of VEGF, suggesting that receptors have an intrinsic ability to dimerize in the absence of ligand [28]. It is thus possible that an unliganded but predimerized pool of VEGFR-2 is activated in a Rho-dependent manner, leading to the increase in phosphorylation of the receptor and the concomitant recruitment of tyrosine phosphorylated proteins observed in the present study.

The Rho-dependent pathways leading to VEGFR-2 phosphorylation and stimulation of its kinase activity remain to be characterized. The involvement of geranylgeranylated proteins in the modulation of growth-factor-receptor tyrosine phosphorylation activities has recently been suggested, based on the selective inhibition of the ligand-dependent tyrosine phosphorylation of both platelet-derived growth factor (PDGF) and epidermal growth factor ('EGF') receptors by inhibitors of isoprenylation [29]. Since post-translational modification of Rho proteins by geranylgeranylation is essential for their association with cellular membranes and biological activities [12], the present results suggest that RhoA could represent the geranylgeranylated protein responsible for the control of the tyrosine phosphorylation activities of these receptors. This hypothesis is supported by our observation that the overexpression of a dominant-active but non-isoprenylable mutant of RhoA (RhoAQ63LC190R) failed to stimulate the tyrosine phosphorylation of VEGFR-2.

It has been demonstrated that RhoA associates physically with the PDGF receptor upon stimulation of the cells with PDGF [30], but we have been unable to detect a similar recruitment of RhoA to VEGFR-2 upon stimulation of endothelial cells with VEGF or CNF1 (D. Gingras and R. Béliveau, unpublished work). This suggests that additional proteins might induce the activation of the receptor either by inducing conformational changes in the protein or by directly phosphorylating specific tyrosine residues within the cytoplasmic tail of the receptor. In this context, it is noteworthy that recent studies have suggested that full activation of the VEGFR-2 and PDGF receptors involves the phosphorylation of multiple tyrosine residues within the receptors, possibly by other protein kinases [31,32].

In summary, our results suggest that Rho proteins play an important role in VEGFR-2 function by modulating the Tyr(P) content and kinase activity of the receptor. Further studies aimed at the identification of the Rho-dependent signalling pathways leading to the activation of VEGFR-2, and their involvement in the signalling cascade triggered by this angiogenic cytokine, may be of significant importance in the understanding of the mechanisms involved in tumour angiogenesis.

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