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c-Jun Kinase Mediates Expression of VEGF Induced at Transcriptional Level by Rac1 and Cdc42Hs but not by RhoA

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Abstract Tumour angiogenesis is mediated by increased levels of vascular endothelial growth factor (VEGF). We have studied the mechanism by which endogenous activation of Rho oncoproteins regulates VEGF expression in COS-7 and NIH3T3 cells. We carried out transient and stable transfection with constitutively activated rhoA, rac1, and cdc42 mutants in COS-7 and NIH3T3 cells, respectively in the absence of external stimuli. Western blot and inmunohistochemistry assays of those cells revealed increased VEGF protein expression. Cotransfection with constitutively activated rhoA, rac1, and cdc42 mutants and a VEGF promoter-reporter construct showed an increase in VEGF promoter transcriptional activity induced by Rho oncoproteins in COS-7 and NIH3T3. c-Jun kinase had been described as a MAPK involved in Rho oncoproteins pathways. Interestingly, we found that c-Jun kinase chemical inhibition as well as transient transactivation assays using dominant negative c-Jun kinase mutant abolished the VEGF promoter transcriptional induction by Rac1 and Cdc42 but not by RhoA. These findings indicate that Rho oncoprotein endogenously activated regulates VEGF expression through a transcriptional mechanism, and that the c-Jun kinase activity is a mediator in the expression of VEGF induced by Rac1 and Cdc42 oncoproteins, but not of that induced by RhoA. J. Cell. Biochem. 98: 650–660, 2006.

Key words: RhoA; Rac1; Cdc42; VEGF; JNK

VPF/VEGF is a potent angiogenic factor. VEGF is uniquely able to stimulate angiogenesis directly, it acts as a potent vasodilator, and it is able to increase vascular permeability [Ku et al., 1993; Bates and Curry, 1996; Ferrara and Bunting, 1996]. VEGF is expressed in nearly all cell types, but malignant tumour cells show overexpression of VEGF and this expression is closely associated with the induction and maintenance of the neovasculature in diverse tumours [Rak et al., 1995; FitzGerald et al., 2004]. The expression of VEGF may be induced by diverse agents such as IL-1β, IL-6, TGFβ, PDGF-B, bFGF, and IGF-I [Stavri et al., 1995; Cohen et al., 1996; Punglia et al., 1997; Akagi et al., 1998; Berse et al., 1999; Miele et al., 2000; Jung et al., 2001; Fukuda et al., 2002; Kotsuji-Maruyama et al., 2002; Gruden et al., 2003; Menu et al., 2004]. Additionally, external stimuli, such as hypoxia, can induce VEGF expression [Kourembanas et al., 1998; Bussink et al., 2003; Choi et al., 2003].

Oncogenic Ras regulates angiogenic signaling through enhanced VEGF secretion in diverse cell lines [Kranenburg et al., 2004]. Some authors have found increased VEGF transcription induced through the Ras > Raf > MEK1 > MAPK kinase cascade and Ras > PI(3)K > PDK > PKB signaling pathway [Mazure et al., 1997; Milanini et al., 1998; Sodhi et al., 2001]. Also, Ras may modulate VEGF mRNA stabilization via Ras > Rac1 > MEKK1 > JNK signaling pathway [Pages et al., 2000]. Likewise, Ras-enhanced VEGF translation initiation mediated by PI(3)K > PDK > PKB > FRAP and

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Raf > MEK > ERK1/2 signaling routes [Gingras et al., 1999; Herbert et al., 2002].

RhoA, Rac1, and Cdc42Hs oncoproteins are small GTPases members of Rho family proteins that belong to the Ras superfamily. They fluctuate between an active GTP-bound state and an inactive GDP-bound state [Aznar and Lacal, 2001]. Physiologically RhoA, Rac1, and Cdc42Hs are involved in the regulation of several important biological processes such as cell growth and differentiation, cell cycle, cytoskeleton regulation, membrane trafficking, apoptosis, stress response, cellular adhesion, apoptosis, and development [Ridley, 1999; Blanchard, 2000; Aznar and Lacal, 2001; Braga, 2002]. It has also been suggested that these Rho proteins play a role in angiogenesis. In this way, RhoA signal transduction pathway suppression by C3 exoenzyme or by p160ROCK inhibitor abolished angiogenesis in HUVEC cells and in vivo assays on the chorioallantoic membrane [Uchida et al., 2000]. In addition, external stimuli such as hypoxia enhanced VEGF and HIF-1 mRNA levels mediated by RhoA activation [Hirota and Semenza, 2001; Turcotte et al., 2003]. Rac1 also mediated the overexpresssion of HIF-1 in response to hypoxia [Hirota and Semenza, 2001]. However, the mechanism by which Rho oncoproteins regulate VEGF expression remains unknown.

Numerous effectors and signaling pathways activated by RhoA, Rac1, and Cdc42Hs have been identified [Benitah et al., 2004]. The c-Jun kinase-signaling pathway is one of the routes activated by Rho proteins. The activity of c-Jun kinase enzyme is dependent on Rac1 and Cdc42 but not on RhoA in NIH3T3 cell line [Coso et al., 1995]. Although the c-Jun kinase enzyme has been involved in a post-transcriptional regulation mechanism stabilizing the VEGF mRNA [Pages et al., 2000], the role of c-Jun kinase in the VEGF expression mediated by Rho oncoproteins has not been studied.

Here we studied the roles of RhoA, Rac1, and Cdc42 and the mechanisms by which they may induce VEGF expression, as well as the involvement of c-Jun kinase in Rho-VEGF pathway.

MATERIALS AND METHODS

Plasmids

pCEFLAU5, expression plasmids of constitutively activated mutants of Ras and RhoA, Rac1, and Cdc42 proteins (pCEFLAU5-rasVal12, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL) and expression plasmids of dominant negative mutants of Ras, RhoA, Rac1, Cdc42Hs, and JNK (pCEFLAU5-rasDN, pCEFLAU5-rhoADN, pCEFLAU5-cdc42DN, and pCEFLAU5-JNKDN) were kindly provided by Dr. S. Gutkind (NIH, Bethesda). A 1.7 kb of rat 5′-flanking sequence (EcoRV-PstI) containing the VEGF promoter was kindly provided by Dr. A. Adamis (Laboratory for Surgical Research, Children's Hospital, Harvard Medical School, Boston, MA).

Antibodies

Monoclonal human antibody to VEGF (human VEGF is 93% homologous compared with mouse VEGF. Rabbit policlonal antibody to RhoA, mouse monoclonal antibody to Rac1, and mouse monoclonal antibody to Cdc42Hs were kindly provided by Dr. J.C. Lacal (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). Mouse monoclonal antibody to α-actin was kindly provided by Dr. A. Porras (Universidad Complutense, Madrid, Spain).

Cell Lines and Culture Conditions

COS-7 and NIH3T3 cell lines were cultured and maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% foetal calf serum for COS-7 or 10% neonatal calf serum for NIH3T3, at 37°C in 5% CO_2 and 95% air.

Stable Transfection of NIH3T3 Cell Line

NIH3T3 cell line was transfected with pCEFLAU5-rasVal12, PCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, or pCEFLAU5-cdc42QL by electroporation at 0,4 kV and 500 μF with 1 μg of corresponding DNA per million of cells. To obtain stable transfected cell lines, the cells were selected after 2 days of transfection by incubation with 100 μg of neomycin per milliliter of medium.

Transient Transfection Assay

The 1.7 kb fragment of rat VEGF promoter (EcoRV-PstI) was subcloned into the eukaryotic expression vector pd2-eGFP to obtain the green fluorescence gene (*gfp*) under the control of VEGF promoter (pVEGFP-gfp). Cells were transfected by electroporation with 2 μg of plasmid DNAs per million of COS-7 cells and 1 μg of corresponding plasmid DNAs per million

of NIH3T3. Electroporation was carried out using a Bio-Rad apparatus at 0.4 kV and 500 μF. Cells were cultured in 25-ml flasks in growth medium at 37°C overnight and then incubated 24 h in serum-free medium and treated with 10% serum for 4 h only in the cited experiments.

Protein Extraction and Inmunoblotting

After transient transfection of stably transfected cells, cells were maintained in the culture medium for 24 h. After this period, the medium was replaced by a serum free medium for 24 h. Finally, the cells were incubated with 5 μg/ml Brefeldin A for an additional 45-min period. Brefeldin A is a compound that blocks VEGF release from the cells. The protein extracts from transfected COS-7 and NIH3T3 cells were obtained by incubation for 20 min in Lysis Buffer containing 25 mM Tris-phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM Triton X-100, 1% DTT, 15% glicerol, 2 μM Lupeptine, 2 μg/μl proteinin, and 100 μM PMSF. Cell extracts were centrifuged at 6.000 r.p.m. for 3 min. Supernatans were transferred to clean tube, protein concentration was determined by Bradford's method (Bio-Rad) and 30 μg of total protein was loaded in 10% denaturing polyacrilamide gels. Proteins were transferred to PVDF membranes (Schleicher & Schuell) by electrotransfer and blocked with 5% non-fat milk for 2 h. Blots were incubated with anti-VEGF, anti-Ras, anti-RhoA, anti-Rac1 anti-Cdc42, and anti-α-actin antibodies followed by peroxidase-labeled anti-mouse or antirabbit IgG. Developing was performed using the enhanced chemiluminiscence system (Amershan).

Quantitative Real-Time RT-PCR (Q-RT-PCR)

Stably transfected cells were grown in culture medium for 24 h. After this, cells were trypsinazed, and washed with PBS. Total RNA was extracted and purified with the eukaryotic Perfect RNA mini-kit (Eppendorf) according to the manufacturer's guidelines. Contaminating genomic DNA was removed by treatment with RNase-free DNase (Roche) for 1 h at 30°C. First-strand cDNA was synthesized at 25°C for 10 min, followed by 42°C for 1 h, 99°C for 5 min, and 4°C for 5 min, using 1 μg of RNA, oligo-dT primers, and the First-strand cDNA synthesis kit for RT-PCR (Roche), according to manufacturer's instructions. As a negative control for genomic DNA contamination, each sample was submitted to the same reaction without reverse transcriptase. The primers used to detect the human *rhoA, rac1, cdc42hs*, and β-*actin* were:

Primers	Sequence	Size cDNA amplification (bp)
VEGF1-forward	5′-ACG CAC TCC AGG GCT TCA TCG TTA CAG-3′	156
VEGF2-reverse	5′-CAT GGA CGT CTA CCA GCG AAG CTA CTG-3′	
βACTIN-forward	5′-TGA GGA GCA CCC TGT GCT-3′	143
βACTIN-reverse	5′-CCA GAG GCA TAC AGG GAC-3′	

Real-time PCR was performed within an iCycler PCR thermocycler (Bio-Rad) and SYBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Bio-Rad) in 20 μl total volume containing SYBR Green Mix (Bio-Rad) and cDNA corresponding to 100 ng of total RNA. Mouse β-*actin* was used in parallel for each run as internal control. Amplification conditions were: 95°C for 5 min; 40 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 1 min; and 72°C for 10 min. The relative level of expression of the *rho-genes* gene was calculated as the ratio of the extrapolated levels of expression of *rho-genes* and β-*actin* mRNAs. The amplification PCR products were analyzed in agarose gel electrophoresis.

Fluorescence Assay

Transfected cells were trypsinized, and washed with PBS. Fluorescence from 3×10^5 cells was measured, in triplicate, using a fluorimeter. Transfection efficiencies were routinely corrected by the ratio of the fluorescence and the β-galactosidase activities in the same sample.

Immunohistochemistry

Stably transfected NIH3T3 cell lines were grown in cover slides and they were incubated for 24 h with DMEM 10% NCS. Afterwards cells were washed with PBS and incubated for 18 h with serum-free media. Cells were treated with 10 μM c-Jun kinase inhibitor II (purchased from Calbiochem) for an additional 6-h period and finally, the cells were incubated with 5 μg/ml Brefeldin A for 45 min. Cells were washed in PBS (0,01M, pH7,4) twice for 5 min and were fixed for 20 min in a 4% paraformaldehide fix

solution in PBS at room temperature and hydrated through graded ethanol steps. Cells were briefly rinsed in PBS and blocked at room temperature using TBSA-BSAT (10 mM Tris, 0.9% NaCl, 0.02% sodium azide, 2% bovine serum albumin, and 0.1% Triton-x100 detergent). Cells were incubated overnight at room temperature with primary antibody at 1:35 dilution. Following rinsing, cells were incubated for 5 h with anti-mouse Cy3 secondary antibody (Jackson Labs) diluted in TBSA-BSAT (1:100). Control sections included omission of the primary antibody. Nuclear staining was performed using DRAQ-5^{TM} (Red Fluorescen Cell-Permeable DNA probe, Biostatus Limited, UK). Immunofluorescence analysis was performed using a Leica TCS SL confocal microscope (Leica LCS Version 2.0).

RESULTS

Constitutively Activated RhoA, Rac1, and Cdc42Hs Oncoprotein Overexpression Induces VEGF Expression

In order to test VEGF expression induction by RhoA, Rac1, and Cdc42Hs, COS-7 cells were transiently transfected with pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, and pCEFLAU5-cdc42QL plasmids to overexpress RhoALeu63, Rac1Leu61, and Cdc42Leu61 mutant proteins, respectively. These mutant proteins are constitutively activated independently of external or internal signals. Rho oncoprotein overexpressions were confirmed by Western blot in COS-7 transient transfected cells (Fig. 1a). Transient rho transfected COS-7 cells showed increased VEGF protein levels as

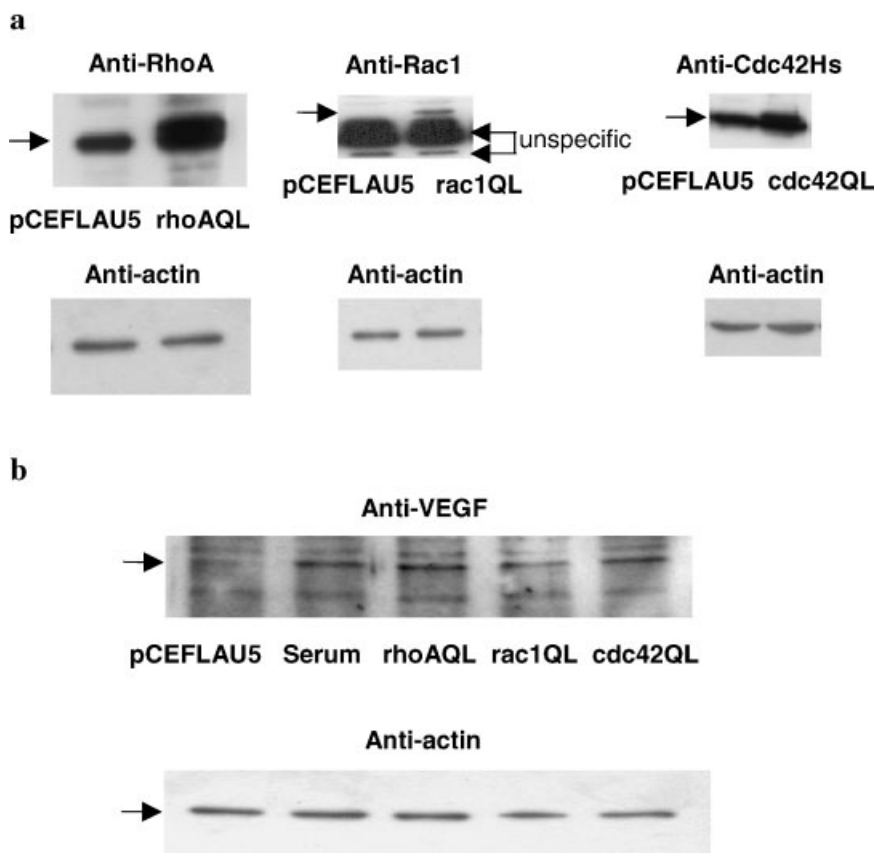

Fig. 1. **a**: RhoA, Rac1, and Cdc42Hs protein overexpression in transient transfected COS-7 cell line. Cells were transfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL. Cells were incubated for 24 h in serum-containing medium and then changed to serum-free medium for 24 h. Cell extracts were obtained and inmunotransferred onto PVDF membrane. Protein levels were analyzed by inmunofluorescence using the corresponding antibodies. **b**: Overexpression of VEGF protein in COS-7 cell line transiently transfected with pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Also, a positive control of cells incubated with serum-containing media was included. After transfection, cell extracts were prepared and analysed by Western blot using anti-VEGF antibody. Western blot using anti-actin antibody was performed as an internal control to correct for loading differences.

compared with control cells transfected with an empty vector (Fig. 1b).

Moreover, pCEFLAU5rhoAQL, pCEFLAU5rac1QL, or pCEFLAU5cdc42QL stable transfection of NIH3T3 cells showed high levels of RhoA, Rac 1, or Cdc42Hs proteins, respectively (Fig. 2a), as well as similarly high VEGF protein levels with respect to control cells (Fig. 2b). Inmunohistochemistry assays in stably transfected NIH3T3 cells with activated mutants of RhoA, Rac1, and Cdc42 showed a clear increase in VEGF protein accumulation in cytoplasm of cells transfected with RhoA, Rac1, and Cdc42 compared with control cells (Fig. 2c). Quantification of mRNA in the same cells by Q-RT-PCR assays showed that Rho-oncoproteins induced a significant increase of the VEGF mRNA levels (Fig. 2d).

RhoA, Rac1, and Cdc42 Induce the Transcriptional Activation of the VEGF Promoter

To analyze a VEGF transcriptional regulatory mechanism by Rho oncoproteins the VEGF promoter was cloned into pd2-eGFP to obtain the green fluorescence protein expressed under the control of VEGF promoter

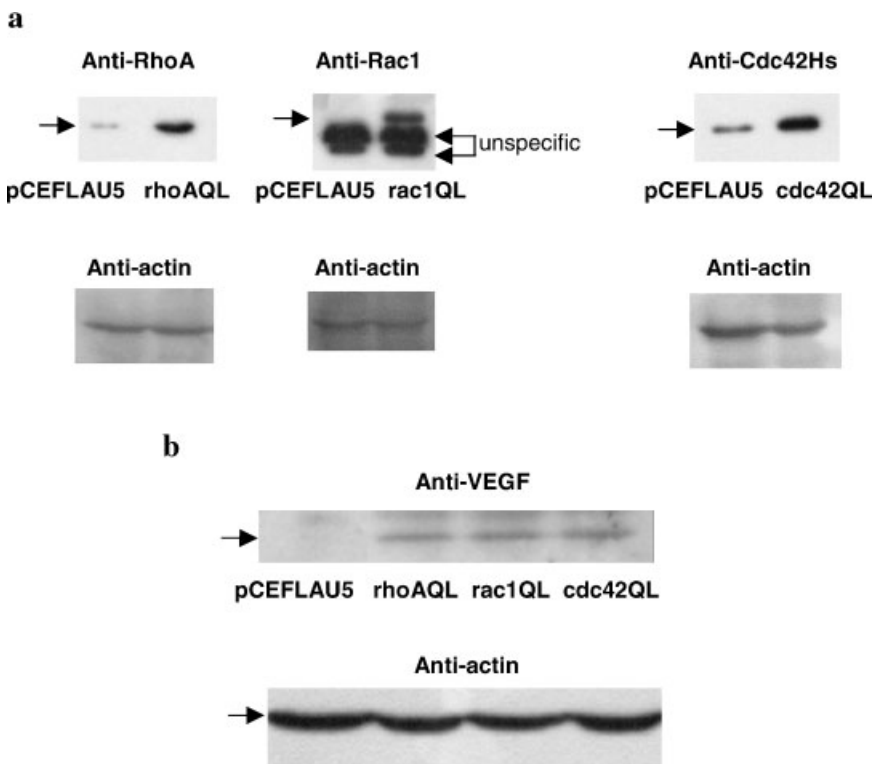

Fig. 2. **a**: RhoA, Rac1, and Cdc42Hs protein overexpression in stably transfected NIH3T3 cells. Cell cultures overexpressing RasVal12, RhoAQL, Rac1QL, or Cdc42QL were incubated with serum-free medium for 24 h. Cell extracts were obtained and inmunotransferred onto PVDF membrane. Protein levels were analysed by inmunofluorescence using the corresponding antibodies. **b**: Overexpression of VEGF protein in NIH3T3 cell line stably transfected with pCEFLAU5-rasVal12, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Stably transfected NIH3T3 overexpressing RhoA, Rac1, and Cdc42Hs was incubated for 24 h with serum-free medium. Afterwards, cells were incubated with Brefeldin A for an additional 1-h period. Cell extracts were prepared and analysed by Western blot using anti-VEGF antibody. Western blot using anti-actin antibody was performed as an internal control to correct for loading differences. **c**: Overexpression of VEGF protein in NIH3T3 cell line stably transfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Stably transfected NIH3T3 overexpressing RhoA, Rac1, and Cdc42Hs and control cells was incubated for 24 h with serum-free medium. Afterwards, cells were incubated with Brefeldin A for 1 h. Cells were prepared and analyzed by inmunohistochemistry assay using anti-VEGF antibody. **d**: Overexpression of VEGF transcripts in NIH3T3 cell line stably transfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Stably transfected NIH3T3 overexpressing RhoA, Rac1, and Cdc42Hs and control cells was incubated for 24 h with serum-free medium. Afterwards, cells were incubated with Brefeldin A for 1 h. RNA was obtained from the VEGF mRNA levels analyzed by quantitative real-time RT-PCR in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

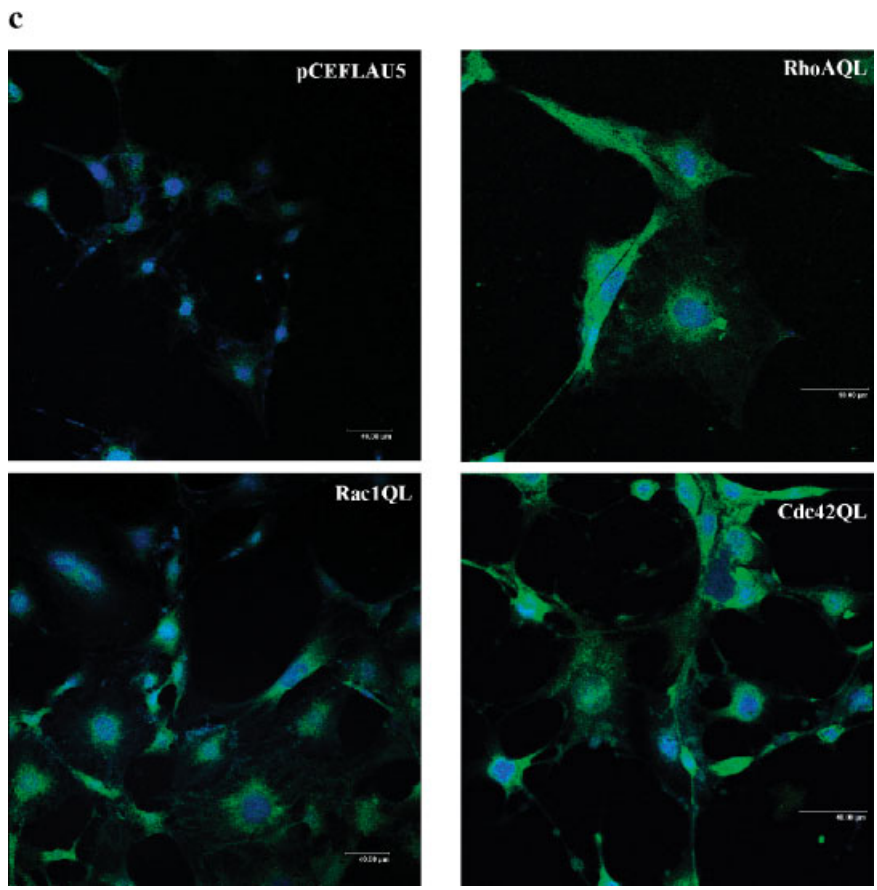

Fig. 2. (*Continued*)

(pVEGFP-gfp). In order to test whether RhoA, Rac1, and Cdc42 induce transcriptional activation of the VEGF promoter, pVEGFP-gfp was transiently cotransfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, or pCEFLAU5-cdc42QL into COS-7 and NIH3T3. We found a four- to five-fold increase of GFP expression in COS-7 cells and a lower 2.5–3.5 fold increase in NIH3T3 cells, after overexpression of constitutively activated mutants of rhoA, rac1, or cdc42 (Fig. 3a,b, respectively). Therefore, transient overexpression of RhoA, Rac1, and Cdc42Hs oncoproteins induces VEGF expression by transcriptional activation of the VEGF promoter in NIH3T3 and COS-7 cell lines.

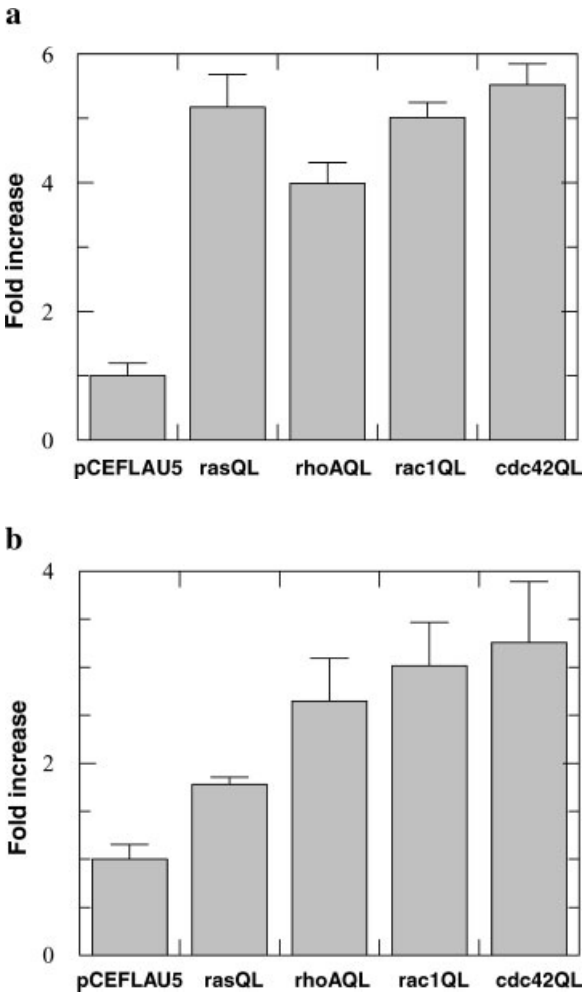

Fig. 3. **a**: RhoA, Rac1, and Cdc42 proteins enhance VEGF promoter activity in COS-7 cells. COS-7 cell line was transfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, or pCEFLAU5-cdc42QL, pVEGFP-gfp and pCMV-β-galactosidase. Results are expressed as factor of increase considering as one the fluorescence of cells transfected with pCEFLAU5. VEGF activation by serum was used as a positive control. The data represent three experiments performed in triplicate. **b**: Ras, RhoA, Rac1, and Cdc42 proteins enhance VEGF promoter activity in NIH3T3 cells. NIH3T3 cell line was transfected with pCEFLAU5, pCEFLAU5-rasVal12, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL or pCEFLAU5-cdc42QL, pVEGFP-gfp, and pCMV-β-galactosidase. Results are expressed as factor of increase considering as one the fluorescence of cells transfected with the empty vector. VEGF activation by RasVal12 was used as a positive control. The data represent two experiments performed in triplicate.

c-Jun Kinase is a Mediator of the VEGF Promoter Transactivation Induced by Rac1 and Cdc42Hs but not by RhoA

To test whether c-Jun Kinase acts a mediator in the VEGF promoter transactivation induced by RhoA, Rac1, and Cdc42Hs; COS-7 cells were cotransfected with pVEGFP-gfp, the dominant negative mutant of c-Jun Kinase (pJNKDN) and one of the Rho overexpression plasmids (pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, or pCEFLAU5-cdc42QL). Fluorescence assays demonstrated that VEGF promoter activation by Rac1 and Cdc42Hs was blocked by the c-Jun Kinase dominant negative mutant (Fig. 4a). However, the dominant negative mutant of c-Jun kinase did not block the VEGF promoter activation induced by RhoA (Fig. 4a). The level of inhibition of the c-Jun kinase negative dominant mutant was as high as 90% for Rac1 induction and 70% for Cdc42 induction. However, only 10% inhibition of RhoA induction was observed. To confirm this result, stably transfected NIH3T3 cells, which overexpressed one of the activated oncoproteins RhoA, Rac1, and Cdc42 were treated with the c-Jun kinase inhibitor II and the effect of this chemical inhibition was analyzed by Western blot (Fig. 4b) and inmunohistochemistry (Fig. 4c). This c-Jun kinase inhibitor II blocked the VEGF protein expression induced by Rac1 and Cdc42 but did not block the VEGF protein expression induced by RhoA (Fig. 4b,c).

DISCUSSION

VEGF expression is induced by a number of external stimuli as hypoxia [Levy et al., 1997; Choi et al., 2003]. Rho oncoproteins have been described as a mediator in the VEGF-induction pathway [Turcotte et al., 2003]. In this study we analyze the role of RhoA, Rac1, and Cdc42Hs oncoproteins in the induction of VEGF expression, as well as the mechanism involved.

We found in Western blots, Q-RT-PCR experiments, and inmunohistochemistry data that constitutively activated mutants of RhoA, Rac1, and Cdc42Hs increase VEGF expression. This was obseved after transient transfection of COS-7 cells with activated oncogenes rhoAQL, rac1QL, and cdc42QL, and after stable transfection of NIH3T3 cells. Data from Q-RT-PCR assays of VEGF mRNA levels were unexpectedly high for RhoA, however the VEGF promoter it is activated to a similar extend by the three oncoproteins (Figs. 1b and 2b). Post-transcriptional mechanisms of regulation such as mRNA stability and translation regulation might overlap the transcriptional regulation of this promoter, as it has been described in the activation of VEGF by *ras* [Kranenburg et al., 2004]. Previous studies have shown that low-oxygen

conditions activated RhoA, which increased HIF-1 and VEGF expression [Turcotte et al., 2003; Hayashi et al., 2005]. Our results, obtained in the absence of any external stimuli, confirm the role of RhoA in VEGF-induced expression. In addition, we found a similar effect produced by Rac1 and Cdc42 oncoproteins. Therefore, oncogenic RhoA, Rac1, and Cdc42Hs may regulate angiogenic signaling through enhanced VEGF secretion.

In order to know the mechanism by which Rho oncoproteins mediate VEGF expression we analyzed whether this occurs at transcriptional level. We used a VEGF promoter-gfp reporter system and found increased VEGF promoter activity after RhoA, Rac1, and Cdc42 overexpression in both COS-7 and NIH3T3 cell lines. Other authors found high VEGF mRNA levels after RhoA activation [Turcotte et al., 2003]. Our results indicate that RhoA, Rac1, and Cdc42Hs induced VEGF by a transcriptional mechanism.

Previous experiments indicate that both Rac1 and Cdc42Hs activate c-Jun Kinase, but not RhoA [Coso et al., 1995]. Also, previous reports showed that the c-Jun kinase transcription factor is involved in the activation of transcription factors involved in cellular response to hypoxia [Alfranca et al., 2002]. In addition it has been demonstrated that the activation of c-Jun kinase is crucial for the increase of VEGF mRNA stabilization mediated by stress protein kinases [Pages et al., 2000]. In order to ascertain if c-Jun kinase is involved in the VEGF transcriptional regulation induced by overexpression of activated rhoA, rac1, and cdc42 mutants, we performed co-transformation with a dominant negative mutant of c-Jun kinase

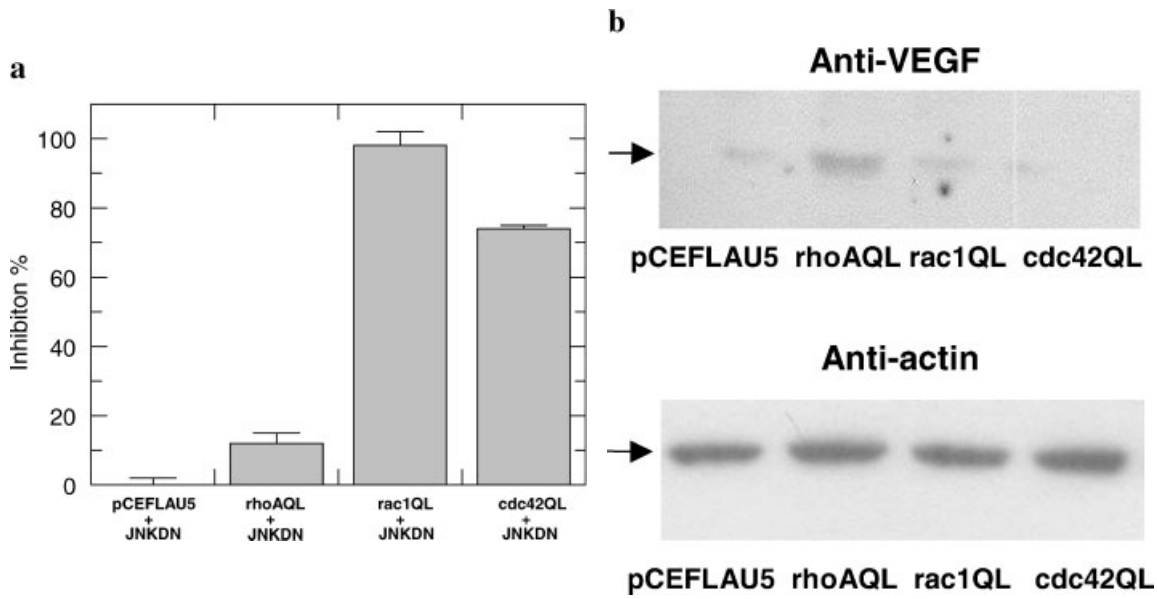

Fig. 4. **a**: The negative dominant mutant of c-Jun kinase abolishes the VEGF promoter transactivation induced by Rac1 and Cdc42 in COS-7 cell line. COS-7 cell line was transfected with pCEFLAU5, pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL or pCEFLAU5-cdc42QL, pVEGFP-gfp, pCMV-β-galactosidase, and with the c-Jun kinase negative dominant mutant or the empty vector. Results are expressed as the inhibition percentage, considering as 100% of activation the fluorescence of corresponding cells transfected with the corresponding Rho expression vector alone without c-Jun kinase dominant negative expression vector. The data represent two experiments performed in triplicate. **b**: Inhibition of overexpression of VEGF protein by c-Jun kinase inhibitor II in NIH3T3 cell line stably transfected with pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, and pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Stably transfected NIH3T3 overexpressing RhoA, Rac1, and Cdc42Hs were incubated for 18 h with serum-free medium. Afterwards, cells were incubated with c-Jun kinase inhibitor II for 6 h and with Brefeldin A for an additional 1-h period. Cell extracts were prepared and analyzed by Western blot using anti-VEGF antibody. Western blot using anti-actin antibody was performed as an internal control to correct for loading differences. **c**: Inhibition of overexpression of VEGF protein by c-Jun kinase inhibitor in NIH3T3 cell line stably transfected with pCEFLAU5-rhoAQL, pCEFLAU5-rac1QL, and pCEFLAU5-cdc42QL. Control cells were only transfected with empty vector pCEFLAU5. Stably transfected NIH3T3 overexpressing RhoA, Rac1, and Cdc42Hs and control cells were incubated for 18 h with serum-free medium. Afterwards, cells were incubated with c-Jun kinase inhibitor II for 6 h and with Brefeldin A for 1 h. Cell extracts were prepared and analyzed by Inmunohistochemistry assay using anti-VEGF antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

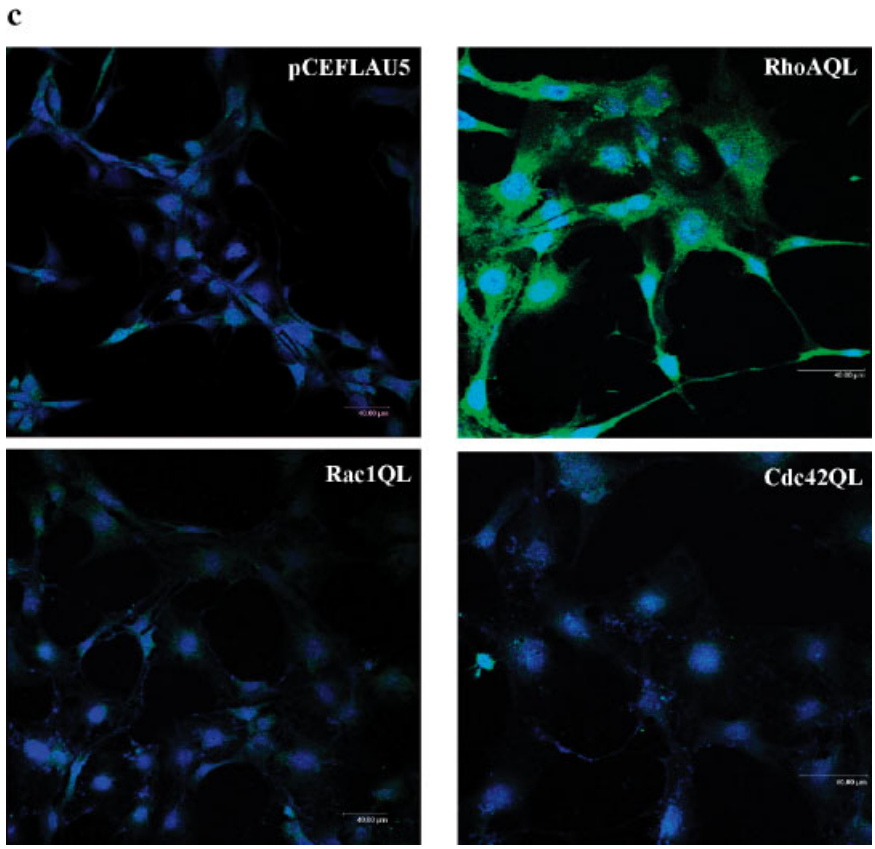

Fig. 4. (*Continued*)

and some of the rho-activated oncogenes. The c-Jun kinase dominant negative mutant abolished the VEGF promoter activity induced by Rac1 and Cdc42Hs, but it had no effect on the VEGF transcriptional induction by RhoA. Therefore, we found that c-Jun kinase is required for the VEGF promoter activation induced by Rac1 and Cdc42Hs. In addition, Western blot and inmunohistochemistry analysis showed that the c-Jun kinase inhibitor II blocked the increases of VEGF protein expression induced by Rac1 and Cdc42Hs but not by RhoA. These data reveal an essential role of c-Jun kinase in the pathway involved in VEGF expression mediated by Rac1 and Cdc42. The decreased levels of VEGF protein observed in presence of the c-Jun kinase inhibitor II in cells overexpressing constitutively activated Rac1 and Cdc42 mutants are consistent with JNK-dependent transcriptional activation. However, we cannot exclude VEGF mRNA stabilization by c-Jun kinase previously described by Pages. Consequently our results show that c-Jun kinase also regulates the level of VEGF transcription in response to Rac1 and Cdc42Hs. Although the role of Rac1 and Cdc42 in VEGF expression was previously described [Turcotte et al., 2003], it was unknown the pathway by which this activation occurs. However, it had been established that RhoA promotes the expression of VEGF through HIF1-α induction [Turcotte et al., 2003]. Therefore, VEGF transcriptional induction by Rho oncoproteins proceeds through two pathways: RhoA → HIF1-α → VEGF and Rac1 or Cdc42 → JNK → VEGF.

In this study we have shown that RhoA, Rac1, and Cdc42 induce VEGF protein expression. We have demonstrated that this increase of VEGF expression is due to a transcriptional mechanism. In addition, we have found that c-Jun kinase mediates VEGF transcriptional upregulation induced by Rac1 and Cdc42. Our results have described for first time a new role of c-Jun kinase in VEGF transcriptional activation in response to Rac1 and Cdc42.

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