Both v-Ha-Ras and v-Raf Stimulate Expression of the Vascular Endothelial Growth Factor in NIH 3T3 Cells*

(Received for publication, December 14, 1994, and in revised form, June 7, 1995)

Stefan Grugel, Günter Finkenzeller, Karin Weindel, Bernhard Barleon, and Dieter Marme‡

From the Institute for Molecular Medicine, Tumor Biology Center, Breisacher Strasse 117, D-79106 Freiburg, Germany

Stimulation of NIH 3T3 cells with platelet-derived growth factor (PDGF)-BB and 12-O-tetradecanoylphorbol-13-acetate (TPA) enhances vascular endothelial growth factor (VEGF) gene expression. To address the question of whether Ras and Raf are involved in the induction of VEGF gene expression by PDGF and TPA, we examined the effects of both factors on NIH 3T3 cells stably transfected with v-Ha-ras or v-raf. In serum-starved NIH 3T3 cells, only low levels of mRNA expression could be detected, whereas both ras and raf transformed cell lines express enhanced levels of VEGF mRNA. Positive immunofluorescence signals could be detected in v-Ha-ras or v-raf transformed cell lines but not in unstimulated NIH 3T3 cells. VEGF from conditioned medium of v-raf transformed NIH 3T3 cells was partially purified by chromatography on heparin-Sepharose. Biological activity of this VEGF protein was demonstrated by competition with binding of recombinant 125I-VEGF to human umbilical vein endothelial cells and by its ability to stimulate proliferation of these cells.

Vascular endothelial growth factor (VEGF) has several features as a mediator of both normal and pathological angiogenesis. It specifically stimulates growth of endothelial cells and induces angiogenesis in vivo. VEGF is produced by several differentiated cell types (1–3), and VEGF mRNA is also expressed in various transformed cell lines (7, 8). The VEGF gene is transcribed into at least four mRNA types by alternative splicing, giving rise to four distinct VEGF proteins of 121, 165, 189, and 206 amino acids after cleavage of the 26-amino acid signal peptide (9, 10). The two smaller proteins are efficiently secreted, whereas the larger forms apparently remain associated with heparin-containing proteoglycans on the cell surface (10). Four AP-1 binding sites have been identified within the promoter sequence of the VEGF gene (9). Cells either treated with phorbol ester, leading to activation of protein kinase C (PKC), or stimulated with PDGF-BB induce VEGF gene expression, suggesting the participation of AP-1 binding sites (9, 11). Therefore, PKC is an essential component in signal transduction leading to VEGF expression (11).

Several lines of evidence strongly implicate Ras as an essential component of growth-regulating signal transduction by growth factor receptor protein-tyrosine kinases (12). The c-ras proto-oncogene encodes a 21,000-Da guanine nucleotide-binding protein (13, 14), which is thought to be involved in the regulation of cell growth, differentiation, and carcinogenesis (15–17). When microinjected into quiescent fibroblasts, the Ras protein has been shown to be a potent mitogen (15). In addition, microinjection into pheochromocytoma PC 12 cells has been shown to induce neurite outgrowth (18). Ras also plays an important role in the pathogenesis of several malignancies including tumor growth. Mutations in the ras gene that increase the proportion of Ras in the GTP-bound state relative to Ras-GDP activate the biological function of Ras and have been found in a large number of human tumors (19, 20).

The ras proto-oncogene products are cytoplasmic serine/threonine-specific protein kinases, which are activated by growth factors or phorbol esters (21–23). They transmit the incoming signals to the transcriptional machinery in the nucleus. Expression of constitutively activated Raf-1 and A-Raf trans-activate transcription from the oncogene-responsive element in the polyomavirus enhancer, which contains an AP-1/Ets binding site (24), in a Ras-independent manner (25, 26). In contrast, mitogen- or Ras-induced expression from the oncogene-responsive element was completely dependent on functional Raf (25). Therefore, Raf has been positioned downstream of Ras in the signal transduction pathway leading to proliferation and transformation of NIH 3T3 cells. Recently, direct interaction of normal and oncogenic Ras with Raf has been reported (27, 28). Ras-mediated activation of Raf thus initiates a protein kinase cascade leading to mitogen-activated protein kinase activation and finally to increased AP-1 activity (29, 30).

A pronounced synergism between PKC and mutant p53 in the induction of VEGF expression was previously reported (31). We therefore addressed the question of whether a functional link exists between oncogenic transformation of cells and the expression of the tumor angiogenesis factor, VEGF. This would generate a connection between genetic alterations, which are thought to play a role in the process of tumor initiation, and the process of tumor progression, mediated by enhanced expression of VEGF. For this purpose, we have used NIH 3T3 cell lines transformed by v-Ha-ras or v-raf, the viral forms of the respective cellular oncogenes, which have been found to be frequently activated by mutation or overexpressed in certain human tumors (19, 20, 32–34).

1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2 S. Grugel and U. R. Rapp, unpublished data.
3 J. Lyons, S. M. Storm, and U. R. Rapp, unpublished data.
Ras, Raf, and VEGF Expression

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The v-Ha-ras plasmid pSV2neo/ras and the v-raf-murine sarcoma virus plasmid were prepared as described previously (35, 36).

**Cell Culture**—All cell lines were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin in a 37 °C, 10% CO2 atmosphere.

Northern Blot and Slot-Blot Analysis—Total RNA was prepared as described (37), subjected to electrophoresis on a 1.25% agarose gel containing 2.2 mM formaldehyde according to the surface tension method (38), and transferred to a nitrocellulose membrane (Hybond C-extra, Amersham) by capillary blotting. Blots were hybridized as described (39, 40).

**Western Blot Analysis**—Cells were lysed in TBST buffer (50 mM Tris hydrochloride, pH 7.2, 150 mM NaCl, 0.2% Triton X-100). Insoluble material was removed by centrifugation at 4 °C for 20 min at 12,000 × g. Protein concentrations were determined by the method of Bradford (41). Samples were resolved by SDS-polyacrylamide gel electrophoresis. The gels were electroblotted onto nitrocellulose, and blots were blocked with 5% (w/v) gelatin in TBST buffer and incubated with polyclonal rabbit antiserum against VEGF. After intensive washing with TBST buffer, the blots were incubated with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma).

**Immunofluorescence**—Cells were seeded in chamber slides (Nunc) and cultivated for 24 h. Afterward, the medium was changed to Dulbecco's modified Eagle's medium supplemented with 0.05% bovine calf serum, and cells were incubated for 20 h at 37 °C. After incubation for 1 h at 4 °C to prevent protein export, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min. After washing with phosphate-buffered saline, cells were incubated for 30 min with PTB buffer (0.2% Triton X-100 in phosphate-buffered saline containing 0.1% bovine serum albumin). Then, cells were incubated for 2 h at room temperature with anti-VEGF antibody (K7.16) in the same buffer. Finally, cells were washed three times for 5 min with PTB buffer. Bound VEGF antibody was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma).

**Binding Competition Assay**—Confluent HUVE cells (passage 3) in 24-well plates were incubated with 1.5 ng/ml 125I-VEGF165 and increasing amounts of unlabeled recombinant VEGF165 from SF9 cells or VEGF from conditioned medium. The concentration of VEGF protein in conditioned medium was estimated by comparison with increasing amounts of recombinant VEGF165 in a Western blot analysis. After 3 h at 4 °C, cells were lysed and radioactivity was measured.

**Proliferation Assay**—A suspension of HUVE cells (passage 3) was prepared with endothelial cell basal medium supplemented with 10% fetal calf serum. Cells were seeded into 24-well plates (5000 cells per well). After 24 h, either recombinant VEGF165 from SF9 cells or VEGF protein from conditioned medium (concentration was estimated as described above) were added. On day 4, medium and VEGF were replaced by fresh medium. On day 7, cells were washed, trypsinized, and counted with a couler counter. The experiment was done three times with essentially identical results.

**RESULTS**

Effects of v-Ha-Ras on VEGF Gene Expression—To examine the effects of v-Ha-Ras transformation of NIH 3T3 cells on expression of the VEGF gene, VEGF mRNA was assayed in v-Ha-ras transformed and control NIH 3T3 cells by Northern blot analysis. VEGF mRNA steady state levels in serum-starved cells were significantly increased in v-Ha-ras transformed cells as compared to control cells (Fig. 1). Furthermore, VEGF expression could be induced in both cell lines by TPA and PDGF-BB, as previously reported (11). But after stimulation with the PKC activator TPA, VEGF expression was augmented in v-Ha-ras transformed cells as compared to control cells (Fig. 2), suggesting that v-Ha-Ras and PKC act in an additive manner on the VEGF mRNA level. This effect was not detectable when v-Ha-ras transformed cells were stimulated with PDGF-BB (Fig. 2).

It was previously reported that at least in some cell types...
tumorigenic progression induced by oncogenic ras is associated with increased PKC gene transcription and PKC expression (42–44). Therefore, we have addressed the question of whether the enhanced TPA inducibility of VEGF expression in NIH 3T3 cells transformed by v-Ha-ras may be due to increased PKC levels in the transfected cell line. Western blot analysis using a polyclonal antibody that recognizes PKCα, βI/βII, γ, and ε did not show any difference in PKC expression between the two cell lines (data not shown), indicating that the increased TPA inducibility of VEGF expression in v-Ha-ras transformed cells is not due to increased PKC levels.

VEGF Gene Expression inraf-transfected NIH 3T3 Cells—To address the question whether constitutive activation of Raf leads to alteration in VEGF expression, v-raf transformed NIH 3T3 cells were examined in Northern blot analysis for VEGF mRNA levels. In serum-starved cells, VEGF expression was significantly increased compared to NIH 3T3 control cells (Fig. 1). The time courses for VEGF expression after stimulation with PDGF-BB and TPA revealed an additional increase in VEGF mRNA to the already elevated basal expression in v-raf transformed cells (Fig. 3). In addition, after stimulation with TPA, VEGF expression was intensified, suggesting a combined action of v-Raf and the PKC activator TPA on VEGF expression.

These results suggest that the Raf kinase is involved in the signal transduction leading to VEGF gene expression. Furthermore, it is strongly indicated that constitutive activation of Raf-1 releases the cell for the necessity of external stimuli to induce VEGF expression.

VEGF Protein Expression in ras- or raf-transformed Cell Lines—To confirm the high basal expression of VEGF mRNA, VEGF protein expression in v-Ha-ras or v-raf transformed cells was analyzed by indirect immunofluorescence using affinity-purified VEGF antibodies (Fig. 4). The specific VEGF fluorescence signal in serum-starved unstimulated NIH 3T3 cells was very weak referring to low VEGF protein expression, whereas the signal in the transformed cell lines was significantly increased. To evaluate the specificity of the VEGF antiserum, immunofluorescence studies were also performed with the corresponding pre-immuneserum, and no fluorescence signal could be detected (data not shown).

By expressing VEGF transiently in mammalian cells, it could be shown that only VEGF165 and VEGF121 are efficiently secreted into the medium, whereas the longer forms VEGF189 and VEGF206 are rather cell associated (10). Based on this observation, together with the strong VEGF immunofluorescence in v-Ha-ras and v-raf transformed cells, we partially purified VEGF from medium conditioned by the v-raf transformed cell line. VEGF could be identified in fractions eluted from heparin-Sepharose columns by Western blot analysis (Fig. 5A). The fraction containing the highest amount of VEGF protein was tested for competition with 125I-labeled rVEGF165 in a binding assay (Fig. 5B). The competition was similar to the positive control with rVEGF165, whereas PDGF-BB and bFGF even at a concentration of 100 ng/ml showed no competition at all. Furthermore, VEGF protein isolated from v-raf transformed cells promoted proliferation of HUVE cells similar to rVEGF165 (Fig. 5C), suggesting that VEGF expressed in NIH 3T3 cells is biologically active.

DISCUSSION

The identification of mitogen-activated protein kinase kinase as a substrate of Raf (45) together with the demonstration of direct phosphorylation of c-Jun (46) by mitogen-activated protein kinase extended the signal transduction pathway (receptor kinase, Ras, Raf, mitogen-activated protein kinase, mitogen-activated protein kinase (Ras/Raf pathway)) into the nu-
Ras, Raf, and VEGF Expression

Fig. 5. Partial purification and characterization of VEGF protein from conditioned medium of v-ras transformed NIH 3T3 cells. A, cells were maintained for 72 h in medium supplemented with 0.05% serum. 100 ml of conditioned medium were run through a heparin-Sepharose column, and proteins were eluted with 1 M NaCl and fractionated in 1-ml aliquots. 15 µl of fractions (1–6) were mixed with non-reducing sample buffer and run on a 10% SDS polyacrylamide gel. Western blot analysis was performed with a VEGF-specific polyclonal antibody from conditioned medium of v-VEGF165 expressed in Sf9 cells; M, molecular weight marker. B, competition of VEGF binding. Confluent HUVE cells were incubated with 1.5 ng/ml 125I-VEGF165 and increasing amounts of either unlabeled recombinant VEGF165 (open circles) or VEGF from conditioned medium from v-raf transformed NIH 3T3 cells (closed circles). As negative control, a 100-fold excess of unlabeled PDGF-BB (open triangle) or bFGF (closed triangle) was used. The experiment was done three times with essentially identical results. C, proliferation of HUVE cells. HUVE cells were seeded into 24-well plates and treated with either recombinant VEGF165 (open circles) or VEGF protein from conditioned medium (closed circles). After 7 days, cells were counted with a coulter counter. The experiment was done three times with essentially identical results. In B and C, the amount of purified VEGF was estimated by Western blot analysis.

The enhanced basal expression of VEGF mRNA in serum-starved NIH 3T3 cells transformed by v-Ha-ras or v-raf (Fig. 1) also indicates that the Ras/Raf signal transduction pathway plays an essential role in the regulation of VEGF gene expression. Despite the increased expression in ras- or raf-transformed cell lines, VEGF expression was still further enhanced by treatment of cells with PDGF and TPA (Figs. 2 and 3). This increase of VEGF indicates that a second, Ras- and Raf-independent, signal transduction pathway may exist or, with regard to Raf, that the already constitutively activated Raf kinase becomes additionally positively regulated by phosphorylation of specific sites in the kinase domain after PDGF or TPA stimulation.

The enhanced basal VEGF mRNA expression also resulted in higher VEGF protein expression, as shown by indirect immunofluorescence (Fig. 4). Furthermore, VEGF protein could be isolated from conditioned medium of v-raf transformed cells (Fig. 5A). Characterization of the secreted VEGF unveiled a specific competition to 125I-labeled VEGF165 (Fig. 5B), and it was shown to be biologically active for HUVE cells (Fig. 5C).

Taken together, these results clearly demonstrate the essential role of the Ras/Raf signal transduction pathway in the regulation of VEGF expression in NIH 3T3 cells. This indicates that Ras as well as Raf are not only involved in cell proliferation and differentiation but also in tumor progression, mediated by increased expression of the tumor angiogenesis factor VEGF.

Acknowledgment—We thank Dr. W. Köhl for kindly providing the v-Ha-ras transformed cell line.

REFERENCES

1. Ferrara, N., and Hengel, W. J. (1989) Biochem. Biophys. Res. Commun. 161, 851–858
2. Ferrara, N., Houck, K. A., Jakeman, L. B., Winer, J., and Leung, D. W. (1991) Cell. Biol. 47, 211–218
3. Cooper, D. T. (1991) J. Cell. Biol. 47, 219–223
4. Gasparov, D., Abraham, J. A., and Schilling, J. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7311–7315
5. Ferrara, N., Winer, J., and Barton, T. (1991) Growth Factors 5, 141–148
6. Phillips, H. S., Hains, J., Leung, D. W., and Ferrara, N. (1991) Endocrinology 127, 965–967
7. Senger, D., Pernuzzi, C. A., Feder, J., and Dvorak, H. F. (1986) Cancer Res. 46, 5629–5632
8. Rosenthal, R., Megyesi, J. F., Hengel, W. J., Ferrara, N., and Folkman, J. (1990) Growth Factors 4, 53–59
9. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gasparov, D., Fiddes, J., and Abraham, J. (1991) J. Biol. Chem. 266, 11947–11954
10. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) Mol. Endocrinol. 5, 1806–1814
11. Finkenzeller, G., Marmé, D., Welch, A., and Hug, H. (1992) Cancer Res. 52, 4821–4823
12. Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7026–7029
13. Barbas, M. G. (1987) Annu. Rev. Biochem. 56, 779–827
14. Chardin, P. (1991) Cancer Cells 3, 117–126
15. Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985) Cancer Cells 3, 117–126
16. Vaidya, T. B., Weyman, C. M., Teegarden, D., Ashendel, C. L., and Taparowsky, E. J. (1991) J. Cell Biol. 114, 809–820
17. Zhang, K., de Cluys, J. E., Vass, W. C., Papageorge, A. G., McCormick, F., and Lowy, D. R. (1990) Nature 346, 754–756
18. Satoh, T. S., Nakamura, S., and Kaziro, Y. (1987) Mol. Cell. Biochem. 7, 4553–4556
19. Almoguera, C., Shiba, D., Forrester, K., Martin, J., Arinhein, N., and Perucchini, M. (1988) Cell 53, 549–554
20. Forrester, K., Almoguera, C., Han, K., Grizzle, W. E., and Perucchini, M. (1987)
Ras, Raf, and VEGF Expression

25919

Nature 237, 298–303
21. Rapp, U. R. (1991) Oncogene 6, 495–500
22. Oshima, M., Sithanandam, G., Rapp, U. R., and Guroff, G. (1991) J. Biol. Chem. 266, 23753–23760
23. Stephens, R. M., Sithanandam, G., Copeland, T. D., Kaplan, D. R., Rapp, U. R., and Morrison, D. K. (1992) Mol. Cell. Biol. 12, 3733–3742
24. Wasylyk, C., Wasylyk, B., Heidecker, G., Huleihel, M., and Rapp, U. R. (1989) Mol. Cell. Biol. 9, 2247–2250
25. Bruder, J. T., Heidecker, G., and Rapp, U. R. (1992) Genes & Dev. 6, 545–556
26. Troppmair, J., Bruder, J. T., App, H., Cai, H., Liptak, L., Széberényi, J., Cooper, G. M., and Rapp, U. R. (1992) Oncogene 7, 1867–1873
27. Warne, P. H., Viciana, P. R., and Downward, J. (1993) Nature 364, 352–355
28. Zhang, X. Z., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 308–313
29. Imler, J. L., Schatz, C., Wasylyk, C., Chatton, C., and Wasylyk, B. (1988) Nature 332, 275–278
30. Williams, N. G., Paradis, H., Agarwal, S., Charest, D. L., Pelech, S. L., and Roberts, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5772–5776
31. Kieser, A., Weich, H. A., Brandner, G., Marmé, D., and Köch, W. (1994) Oncogene 9, 963–969
32. Rapp, U. R., Storm, S. M., and Cleveland, J. L. (1987) Hamatol. Bluttransfus. 31, 450–459
33. Rapp, U. R., Huleihel, M., Pawson, T., Linnola, I., Minna, J. D., Heidecker, G., Cleveland, J. L., Beck, T., Forchhammer, J., and Storm, S. M. (1988) J. Int. Assoc. for the Study of Lung Cancer 4, 162
34. Storm, S. M., and Rapp, U. R. (1993) Toxicol. Lett. 67, 201–210
35. Clinton, D. J., Lu, Y. Y., Blair, D. G., and Shih, T. Y. (1987) Mol. Cell. Biol. 7, 3092–3097
36. Rapp, U. R., Golub, S. M., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., and Stephenson, J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4218–4222
37. Chomyczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
38. Rosen, K. M., Lamperti, E. D., and Villa Komaroff, L. (1990) BioTechniques 8, 398–403
39. Weindel, K., Marmé, D., and Weich, H. A. (1992) Biochem. Biophys. Res. Commun. 183, 1167–1174
40. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
41. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
42. Delage, S., Chastrine, E., Empereur, S., Wicott, G., Veissière, D., Capeau, J., Gespach, C., and Cherqui, G. (1993) Cancer Res. 53, 2762–2770
43. Borner, C., Guadagno, S. N., Hsiao, W.-L. W., Fabro, D., Barr, M., and Weinstein, I. B. (1992) J. Biol. Chem. 267, 12900–12910
44. Borner, C., Guadagno, S. N., Hsiao, W.-L. W., and Weinstein, I. B. (1990) Cell Growth & Differ. 1, 653–660
45. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992) J. Biol. Chem. 267, 417–421
46. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 353, 670–674
47. Jänal, S., and Ziff, E. (1990) Nature 344, 463–466
48. Kaibuchi, K., Fukumoto, Y., Oka, N., Hori, Y., Yamamoto, T., Toyoshima, K., and Takai, Y. (1989) J. Biol. Chem. 264, 20885–20888
49. Qureshi, S. A., Cao, X., Sukhatme, V. P., and Foster, D. A. (1991) J. Biol. Chem. 266, 10802–10806
50. Wasylyk, C., Wasylyk, B., Flores, A. C., Begue, A., Leprince, D., and Stehelin, D. (1990) Nature 346, 191–193
51. Gutman, A., Wasylyk, C., and Wasylyk, B. (1991) Mol. Cell. Biol. 11, 5381–5387
52. Köch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D., and Rapp, U. R. (1993) Nature 364, 249–252