VRAP Is an Adaptor Protein That Binds KDR, a Receptor for Vascular Endothelial Cell Growth Factor*

(Received for publication, December 15, 1999)

Li-Wha Wu‡, Lindsey D. Mayo‡, James D. Dunbar‡, Kelly M. Kessler‡, Osman Nidai Ozes‡, Robert S. Warren‡, and David B. Donnen§

From the §Department of Microbiology and Immunology, Indiana University School of Medicine, and Walther Oncology Center, Indianapolis, Indiana 46202 and the ¶Department of Surgery, University of California, San Francisco, California 94143

A protein that binds the intracellular domain of KDR (KDR-IC), a receptor for vascular endothelial cell growth factor (VEGF), was identified by two-hybrid screening. Two-hybrid mapping showed that the VEGF receptor-associated protein (VRAP) interacted with tyrosine 951 in the kinase insert domain of KDR. Northern blot analysis identified multiple VRAP transcripts in peripheral leukocytes, spleen, thymus, heart, lung, and human umbilical vein endothelial cells (HUVEC). The predominant VRAP mRNA encodes a 389-amino acid protein that contains an SH2 domain and a C-terminal proline-rich motif. In HUVEC, VEGF promotes association of VRAP with KDR. Phospholipase C gamma and phosphatidylinositol 3-kinase, effector proteins that are downstream of KDR and important to VEGF-induced endothelial cell survival and proliferative responses, are associated constitutively with VRAP. These observations identify VRAP as an adaptor that recruits cytoplasmic signaling proteins to KDR, which plays an important role in normal and pathological angiogenesis.

Vascular endothelial cell growth factor (VEGF) is an endothelial cell-specific mitogen which directly promotes many events necessary for angiogenesis including the proliferation and movement of endothelial cells, remodeling of the extracellular matrix, the formation of capillary tubules, and vascular leakage (for reviews, see Refs. 1 and 2). VEGF is produced by normal and transformed cells (3, 4) and plays a significant role in the development of the cardiovascular system, the physiology of normal vasculature and pathologies dependent on neo-vascularization, such as diabetic retinopathies, rheumatoid arthritis, and cancer (5–10).

VEGF exerts its actions by binding to cell surface receptor tyrosine kinases, KDR, the human homolog of Flk1, and Flt1 (11–15). Both receptors are structurally similar to members of the PDGF receptor family and consist of an extracellular domain composed of seven immunoglobulin-like motifs, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase that is split by a kinase insert region, and a carboxyl-terminal tail (16). Several studies have shown that Flk1/KDR plays an important role in the proliferation and survival of endothelial cells (15, 17–19). The importance of the VEGF/KDR signaling system is further emphasized by the demonstration that neutralization of VEGF or inhibition of KDR/Flk1 blocks the growth and spread of cancers in animals (20–22).

The role of KDR in promoting endothelial growth and survival together with the observation that this receptor plays an obligate role in the progression of pathologies dependent on angiogenesis led us to search for proteins that might be components of the VEGF/KDR signaling pathway. The approach used was to screen two-hybrid libraries for proteins that directly bind the cytoplasmic domain of KDR (KDR-IC). Because KDR/Flk1 is expressed by hematopoietic cell types, as well as endothelial cells, that share a common lineage (23), human endothelial and B cell two-hybrid libraries were screened. This has led to the identification of the cDNA for an adaptor protein that binds KDR. The VEGF receptor-associated protein, VRAP, contains an N-terminal SH2 domain and a C-terminal proline-rich motif. VRAP mRNA is well expressed in the endothelium, blood cells, liver, lung, and heart. VRAP constitutively binds PLCγ and PI 3-kinase, and stimulation of human umbilical vein endothelial cells (HUVEC) with VEGF results in recruitment of VRAP to KDR. These observations suggest that VRAP is an adaptor protein that shuttles important cytoplasmic effector proteins to KDR such that they can be used to promote VEGF action.

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant VEGF and the cDNA for KDR were gifts from Genentech Inc. (South San Francisco, CA). The human B-cell two-hybrid library was obtained from CLONTECH. Anti-KDR/Flk1 conjugated to agarose and anti-KDR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody directed against PLCγ was from Transduction Laboratories (Lexington, KY). The antibody directed against the 85-kDa regulatory subunit of PI 3-kinase was obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

**Cell Culture and Treatments—**HUVEC obtained as described previously (24) were grown on 0.2% gelatin-coated tissue culture plates in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**Cell Edema Assay—**The inhibition of cell edema by VEGF was measured as described previously (25, 26).

**Cell Proliferation Assay—**HUVEC obtained as described previously (24) were grown on 0.2% gelatin-coated tissue culture plates in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**In Vitro Tube Formation Assay—**A total of 10,000 HUVEC were loaded with 1 μM ethidium dibromide and grown on 0.1% gelatin-coated tissue culture plates in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**Transwell Migration Assay—**HUVEC obtained as described previously (24) were grown on 0.2% gelatin-coated tissue culture plates in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**In Vivo Angiogenesis Assay—**A total of 10,000 HUVEC were loaded with 1 μM ethidium dibromide and grown on 0.1% gelatin-coated tissue culture plates in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**Preparation of Vascular Endothelial Cells—**Endothelial cells were isolated from the umbilical cord of 12 week-old human fetuses (A) and from human umbilical vein (B) as described previously (24, 27). Cells were cultured in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).

**Preparation of Vascular Endothelial Cells—**Endothelial cells were isolated from the umbilical cord of 12 week-old human fetuses (A) and from human umbilical vein (B) as described previously (24, 27). Cells were cultured in endothelial cell basal medium (EBM, Clonetics, Inc.) containing 1% fetal bovine serum. Cytotoxicity assays were performed as described previously (25, 26).
ing, respectively. The intracellular domain of KDR spanning amino acids 788 to 1339 was polymerase chain reaction amplified using Pu polymerase (Stratagene) and subcloned into the SalI and ScaI restriction sites of pGBT9 to produce pGBT9-KDR-IC.

Two-hybrid Library Screening and Evaluation of Protein-Protein Interactions—Two-hybrid assays using the GAL4 system were performed according to the instructions of the manufacturer (CLONTECH). For library screening, Y190 yeast cells were transformed with a human B cell two-hybrid library and pGBT9-KDR-IC. For characterizing interactions of KDR and VRAP, KDR-IC or KDR-IC point mutants in pGBT9 were cotransformed into the SFT526 yeast strain together with VRAP truncation mutants. To prepare truncated versions of VRAP, cDNAs amplified by polymerase chain reaction corresponding to the N-terminal domain (codon 1–94, VRAP-NH2), the SH2 domain (codon 95–186, VRAP-SH2), or the C-terminal proline-rich domain (codon 187–389, VRAP-Pro) were digested with BamHI and EcoRI and subcloned into BamHI/EcoRI-digested pGAD424.

Protein-protein interactions were then identified based on the lacZ phenotype. Site-directed Mutagenesis of KDR—Using pGBT9-KDR-IC as a template, three tyrosine residues (Tyr-951, Tyr-996, and Tyr-1175) were individually mutated to phenylalanine, generating Y951F, Y996F, and Y1175F, respectively. The mutations were effected using the Quick Change Site-directed Mutagenesis Kit (Stratagene). The mutations were verified by DNA sequencing.

RNA Isolation and Northern Blot Hybridization—Poly(A)+ RNA was isolated from low passage (passage 4) HUVEC using Qiagen and Oligotex kits. The RNA was fractionated and then transferred to nitrocellulose membranes. Northern blotting of this and human multiple tissue RNA blots were verified by DNA sequencing.

Immunoprecipitation and Western Blotting—After treatments, HUVEC were washed twice with ice-cold phosphate-buffered saline and lysed by incubation in 50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml leupeptin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate for 30 min at 4 °C. Immunoprecipitations, SDS-PAGE, and Western blotting were then conducted as described previously (25).

RESULTS AND DISCUSSION

To identify proteins involved in KDR signal transduction, KDR-IC fused with the GAL4 DNA binding domain was used as bait to screen human B cell and endothelial cell two-hybrid libraries cloned into the GAL4 activation domain. Using the B cell library, a total of 620,000 colonies were screened. From among eight His "lacZ" colonies isolated, one was a true positive. DNA sequence analysis and data bank searches revealed that the VRAP clone encodes a previously described T cell-specific protein of unknown function (26). VRAP is a 389-amino acid protein comprised of an N-terminal sequence of 94 amino acids, an SH2 domain (amino acids 95–186), and a C-terminal domain (amino acids 187–389) that contains a proline-rich region (Fig. 1A).

The specificity and nature of the interaction between KDR-IC and VRAP was characterized by co-transformation of various constructs into the SFT526 yeast strain, pGBT9 (GAL4-BD) and Lam5' (lamin) were tested for interaction with VRAP and did not activate β-galactosidase. β-Galactosidase activity was detected only in yeast co-transformed with VA3 (p53) and TD1 (the SV40 large T antigen), a positive control, or KDR-IC and VRAP. These results confirm the KDR/VRAP interaction and rule out the possibility that VRAP contains intrinsic transcriptional activity or interacts with other proteins nonspecifically.

Because SH2 domains bind phosphotyrosine, we conducted experiments to determine whether tyrosines in KDR are necessary for interaction with VRAP. Tyrosines corresponding to amino acids 951, 996, 1054, and 1059 in KDR are phosphorylated in bacteria (27) and are likely sites for phosphorylation in mammalian cells. Tyrosines 951 and 996 are in the kinase insert domain of KDR, whereas Tyr-1054 and Tyr-1059 are in the catalytic domain. Tyr-1175 in the C-terminal domain of KDR corresponds to Tyr-1169 in Flt1, a binding site for PLCγ (28, 29). Given that Tyr-951, Tyr-996, and Tyr-1175 are outside of the KDR catalytic domain, these were mutated to phenylalnine to produce Y951F, Y996F, and Y1175F. Using the yeast two-hybrid system, it was found that Tyr-951 in KDR plays an obligatory role in the KDR/VRAP interaction (Fig. 1B). Surprisingly, the VRAP SH2 domain by itself was incapable of interaction with KDR in the two-hybrid system. Further analysis revealed that N- and C-terminal domains of VRAP also did not bind KDR. These observations suggested that each of the domains within VRAP may play a role in sustaining a conformation conducive to receptor binding and that the SH2 domain may have a somewhat unique structure.

This latter supposition was confirmed by a search of GenBankTM, which revealed that the amino acid sequence of the SH2 domain in VRAP is quite different from those in other proteins. Alignment of SH2 domains showed that VRAP shares some homology with the SH2 domains of corkscrew, a protein tyrosine phosphatase, LNK, a cytosolic protein tyrosine kinase, the c-Src kinase and PLCγ, with which it is most closely related.

Northern blot analysis defined the tissue expression pattern

![Image](http://www.jbc.org/Downloadedfromhttp://www.jbc.org/
of VRAP. Multiple tissue blots revealed VRAP mRNA in peripheral leukocytes, the spleen and thymus and also in the heart, lung, and liver, which are well perfused with blood (Fig. 2A). Transcripts of varying sizes which may arise from alternative splicing were present in these tissues and also in RNA from HUVEC (Fig. 2B). VRAP expression was much lower, or nondetectable, in brain, placenta, skeletal muscle, prostate, testis, ovary, small intestine, and colon. Furthermore, using the polyclonal VRAP antibody described below, we detected VRAP in human foreskin fibroblasts (data not shown), as well as HUVEC (see below), indicating that the protein may be expressed in many cell types. Thus, VRAP expression is not restricted to T cells, as first reported (26), but is strongly expressed in blood cell lineages, the endothelium, and other cell and tissue types.

A polyclonal antibody to the proline-rich domain of VRAP was raised in rabbits. The antibody recognized a protein of about 52 kDa in lysates of HUVEC (Fig. 3A). Neutralization of antibody with the immunogen (the proline-rich domain of VRAP) abrogated the ability of anti-VRAP to detect the 52-kDa protein. Having a characterized antibody made it possible to determine whether KDR and VRAP associate. To accomplish this, KDR was immunoprecipitated from control- and VEGF-stimulated HUVEC. As illustrated in Fig. 3B, the blot was reprobed with anti-PLCγ, and VEGF stimulation promotes this process. These observations validate the results derived from two-hybrid screening, which first identified VRAP as a protein that interacts with KDR-IC. Also, the ability of VEGF, which promotes tyrosine phosphorylation of KDR (18), to promote formation of VRAP-KDR complexes provides support for the view that the SH2 domain of VRAP, although somewhat unique, is functionally competent.

The presence of proline-rich motifs in its C-terminal domain led us to test whether VRAP would interact with proteins that contain SH3 domains, among which are PLCγ and PI 3-kinase. These proteins were selected for investigation as we recently showed that, by signaling through KDR, VEGF promotes the tyrosine phosphorylation of phospholipase Cγ (PLCγ), which plays a role in VEGF-induced MAPK activation and endothelial cell proliferation (18). Also activated by signaling through KDR is the Akt serine threonine kinase, a downstream target for phosphatidylinositol 3-kinase and an important cell survival factor (18, 19).

As illustrated in Fig. 4, A and B, Western blot analysis of proteins that co-immunoprecipitated with PLCγ or PI 3-kinase revealed that each effector protein interacts with VRAP. Furthermore, the interactions were constitutive, and the level of association was not augmented by stimulation of HUVEC with VEGF. These observations indicate that VRAP binds cytoplasmic signaling proteins and then acts as a shuttle to bring these into a complex with KDR.

Tyrosine 951 in KDR is a binding site not only for VRAP, but also for PLCγ as well (18), suggesting that PLCγ may complex with KDR directly or through the intermediary of VRAP. It is interesting to consider that competition between VRAP and PLCγ for Tyr-951 may affect the array of other signaling proteins that can be brought into a complex with KDR and thereby...
affect VEGF action. This situation is not unprecedented as the Nck adaptor protein and PI 3-kinase share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor (30). Overall, our observations define VRAP as an adaptor that facilitates and regulates interaction of KDR with effector proteins important to endothelial cell survival and proliferation.

REFERENCES

1. Ferrara, N. (1999) *Kidney Int.* 56, 794–814
2. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, A. (1999) *FASEB J.* 13, 9–22
3. Berse, B., Brown, L. F., Van De Water, L., Dvorak, H. F., and Senger, D. R. (1992) *Mol. Biol. Cell* 3, 211–220
4. Monacci, W. T., Merrill, M. J., and Oldfield, E. H. (1993) *Am. J. Physiol.* 264, C995–C1002
5. Schwieki, D., Itin, A., Neufeld, G., Gitay-Goren, H., and Keshet, E. (1993) *J. Clin. Invest.* 92, 2235–2243
6. Plate, K. H., Breier, G., Weich, H. A., and Risau, W. (1992) *Nature* 359, 521–532
7. Cunningham, S. A., Pia Arrate, M., Brock, T. A., and Waxhm, M. N. (1997) *Biochem. Biophys. Res. Commun.* 240, 635–639
8. Nichishima, R. Li, W. Kashishian, A., Mondino, A., Zhou, M., Cooper, J., Schlessinger, J. (1993) *Mol. Cell. Biol.* 13, 6889–6896
VRAP Is an Adaptor Protein That Binds KDR, a Receptor for Vascular Endothelial Cell Growth Factor
Li-Wha Wu, Lindsey D. Mayo, James D. Dunbar, Kelly M. Kessler, Osman Nidai Ozes, Robert S. Warren and David B. Donner

J. Biol. Chem. 2000, 275:6059-6062.
doi: 10.1074/jbc.275.9.6059

Access the most updated version of this article at http://www.jbc.org/content/275/9/6059

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 13 of which can be accessed free at http://www.jbc.org/content/275/9/6059.full.html#ref-list-1