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Biological Activity and Phosphorylation Sites of the Bacterially Expressed Cytosolic Domain of the KDR VEGF-Receptor

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Summary Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which binds to two structurally similar receptor tyrosine kinases, KDR and FLT1. Towards the goal of clarifying the signal transduction pathways by which VEGF activates endothelial cells, we expressed in bacteria an enzymatically active form of the cytosolic domain of the KDR receptor. The expressed protein undergoes autophosphorylation in both bacterial cells and in its purified form. Using peptide mapping and sequencing of peptides, we identified four tyrosine residues that are phosphorylated corresponding to residues 951, 996, 1054, and 1059 of the KDR protein. The location of the phosphorylated residues in the bacterially expressed protein, and/or the consensus sequences around these sites, suggest they may be identical to the phosphorylated sites of KDR in mammalian cells.

Vascular endothelial growth factor (VEGF) is now well established as a potent activator of angiogenesis and inducer of vascular permeability (1-3). Several effects of VEGF on cultured endothelial cells have been documented including an increase in proliferation rate (1), stimulation of calcium mobilization (4), and potentiation of FGF-induced capillary-like tube formation of cells grown in a collagen matrix (5). There is both a temporal and spatial correlation between VEGF mRNA expression and the onset of angiogenesis during physiological situations requiring neovascularization (6-9). VEGF action

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appears to be rather specific for the endothelium, as high affinity binding sites for VEGF are expressed in vascular endothelial cells but not in most other cell types (10,11).

The DNA encoding two VEGF receptors (KDR/FLK1 and FLT1) have been cloned and expressed (12-14). The two receptor subtypes share many similarities, including tyrosine kinase activity, a kinase insert domain, and seven extracellular immunoglobulin domains. Northern and *in situ* hybridization studies have demonstrated the expression of mRNA for both receptor subtypes in endothelial cells (8,13,15) but the functional significance of each receptor subtype for VEGF action is not clear at present.

The goal of the present study was to identify tyrosine autophosphorylation sites on the KDR receptor subtype. The identification of autophosphorylation sites on other receptor tyrosine kinases (eg. PDGF-R (16,17), EGF-R (18), FGF-R (19)) has proven useful for gaining a clearer understanding as to the earliest steps by which growth factors activate cells. It is expected that similar information for KDR will help clarify signal transduction mechanisms by which endothelial cells respond to VEGF. The approach taken was to express the KDR cytosolic domain (KDR-CD) in bacteria, demonstrate that it has intrinsic tyrosine kinase activity, and then identify the autophosphorylation sites. This strategy has several advantages over using an intact receptor expressed in eukaryotic cells. The bacterial system allows for the isolation of large quantities of protein, and is void of phosphatases and other kinases which make studies using eukaryotic cells more difficult. Furthermore, the approach of expressing the cytosolic domains of receptor tyrosine kinases has proven successful for identifying autophosphorylation sites on both the EGF (18) and FGF (19) receptors.

METHODS

Materials. Monoclonal anti-phosphotyrosine antibody was from Upstate Biotechnology, Lake Placid, NY. Goat anti-mouse secondary antibody and the ECL detection kit were from Amersham, Chicago, Ill. Nitrocellulose membrane was from Bio-Rad Laboratories, Hercules, CA. Trypsin was from Boehringer Mannheim, Indianapolis, IN. Immobilon AV membrane was from Millipore, (Bedford, MA). Potato acid phosphatase, catalogue number P3752, was from Sigma, St. Louis, MO. All other reagents were from standard sources.

Cloning of KDR cytoplasmic domain into the T7 expression system. A 2200 bp BamH1 / EcoR1 restriction fragment of the KDR cDNA (20) was cloned into the bacterial expression vector pET3A (21), the resulting plasmid is designated KDR-CD/pET3A. The KDR-derived cDNA contained the 3'-most 1658 nucleotides of the coding sequence plus approximately 500 bp of 3' untranslated sequence. The 5' end of the KDR-derived sequence is the BamH1 site at nucleotide 2408. This corresponds to amino acid 803 of the native protein (12) which is situated 17 amino acids carboxy terminal to the membrane spanning domain. The 17 amino acids present in the cytosolic domain of the

native receptor but missing in the bacterially expressed cytosolic domain are found within the juxtamembrane portion (22) of the native receptor, and do not contain any of the kinase region.

Expression and purification of KDR-CD. *E. coli* BL21 were transformed with KDR/pET3A using the $CaCl_2$ method (23). A 250 ml culture was induced with 1 mM isopropylthio-β-galactoside (IPTG) for either 4 h at 37°C or for 14 h at 23°C. All subsequent steps were done at 4°C. The cells were pelleted and suspended in 25 ml of 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA and lysed by passage through a French Press. The lysate was centrifuged at 15,000 rpm for 20 minutes in a Beckman J2-21 centrifuge, and both the supernatant (designated cell supernatant) and pellet (designated inclusion body) fractions were saved. The inclusion body fraction was resuspended in 20 ml of 50 mM TRIS.HCl, pH 8.0, 0.1 mM EDTA, 1M NaCl, and 10% glycerol (dialysis buffer). The sample was then dialyzed for 16 hours against dialysis buffer and then dialyzed 4 hours against dialysis buffer containing 200 mM NaCl. The dialyzed material was centrifuged at 15,000 rpm for 15 minutes; the soluble material was saved.

The dialyzed sample was incubated with 1 ml of anti-phosphotyrosine antibody agarose overnight at 4°C. The resin had previously been equilibrated with 20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 100 mM NaCl (wash buffer). The mixture was poured into a column and unbound material collected. The column was washed with 50 ml of wash buffer. Phosphotyrosine containing proteins were eluted by mixing the resin batchwise with 5 ml of wash buffer containing 100 mM phenylphosphate. The eluted material was concentrated to 200 µl using a Centricon 10 microconcentrator (Amicon, Beverly, MA). The sample was diluted to 2 ml with 10 mM sodium phosphate, pH 7.4, 100 mM NaCl, and again concentrated to 200 µl. The cycle of dilution and concentration was repeated two more times in order to reduce the concentration of phenylphosphate in the sample.

KDR-CD from the cell supernatant fraction was purified by incubation of the sample with 1 ml of the anti-phosphotyrosine antibody agarose. Elution of tyrosine phosphorylated protein and concentration of the sample was done as described for the inclusion body fraction.

Western blot analysis of tyrosine phosphorylated proteins. Samples were suspended in 20 µl of gel sample buffer (50 mM TRIS·HCl, pH 6.9, 4% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, and 0.02% bromophenol blue) and boiled for 3 minutes. The samples were loaded onto 7% SDS-polyacrylamide gels, which were run until the dye front was within 1 cm from the bottom. Proteins within the gel were transferred to nitrocellulose; the membrane was incubated for 2 hours in 5% dried milk in TBS-T (100 mM TRIS·HCl, pH 8.0, 150 mM NaCl, 1% TWEEN-20). The membrane was then incubated for 4 hours with 10 ml of anti-phosphotyrosine antibody diluted 1:2,000 in TBS-T. The membranes were washed twice for 10 minutes with TBS-T and then incubated for 1 hour with 10 ml of goat anti-mouse secondary antibody diluted 1:10,000 in TBS-T. Dectection of tyrosine phosphorylated proteins was done using an ECL kit.

Phosphatase treatment of KDR-CD and determination of intrinsic tyrosine kinase activity. Two µl of KDR-CD purified from the cell supernatant was incubated for 30 minutes at 30°C in 10 µl of phosphatase buffer (10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), pH 7.0, 100 mM NaCl, 1 mM $MgCl_2$) and 0.1 U of potato acid phosphatase . Phosphatase activity was inhibited by placing the sample at 4°C and adding 2 µl of 5 mM Na_3VO_4 and 2

µl of 150 mM NaF. The tyrosine kinase activity of the dephosphorylated protein was determined by monitoring autophosphorylation as follows. The protein was incubated at 4°C with 10 mM $MnCl_2$ and either 100 µM ATP or 5 µCi ^{32}P-ATP for periods of time given in the legend to Figure 4. The extent of autophosphorylation was determined using Western blot hybridization or SDS-PAGE autoradiography.

Trypsin digestion of KDR-CD, phosphopeptide mapping, and N-terminal sequencing. KDR-CD, solubilized from the inclusion bodies of bacteria induced with IPTG at room temperature was purified on an anti-phosphotyrosine antibody column. Affinity purified protein (300 µg in 200 µl) was denatured by adding an equal volume of 8M urea, 0.8 M NH_4HCO_3, reduced by incubating with 7 mM DTT at 50°C for 15 min, and then carboxyamidomethylated by incubating with 15 mM iodoacetamide for 15 min at 25°C (24). The sample was diluted to a final urea concentration of 1M prior to adding trypsin at 1:25, w/w, for 18 h at 37°C. Peptides were isolated using a narrowbore HPLC system with a C18 column (Aquapore OD300, 2.1 x 220 mm; Applied Biosystems) and a gradient of acetonitrile (0 to 32% in 100 min, 32 to 80% in 20 min) in 0.1% trifluoroacetic acid.

Tyrosine phosphate containing peptides were detected by spotting 1 µl of each HPLC fraction (diluted 1:10 in 0.5 M potasium phosphate) onto Immobilon AV membrane (Millipore). The membrane was blocked by incubating for one hour in monoethanolamine (10% in 1 M sodium bicarbonate), and then washed 2 times with TBS-T. Incubation of the membrane with anti-phosphotyrosine antibody and detection of positives was done as described above for Western analysis. N-terminal sequence analysis (25) was done using automated Edman degradation with an Applied Biosystems model 477A protein sequencer.

RESULTS

Figure 1 shows that addition of IPTG to *E. coli* BL21 transfected with KDR/pET3A resulted in the expression of a 68 kDa protein. The expression was observed when induction was done at 37°C or at room temperature. By coomassie blue staining, it was determined that the majority of 68 kDa protein was localized to the inclusion bodies of the bacteria (Figure 2). This was true regardless of whether the bacteria were induced at 37°C or at room temperature.

Figure 3 shows the result of Western blot hybridization of bacterially expressed proteins using an anti-phosphotyrosine antibody. The results demonstrate that the KDR-CD was phosphorylated at tyrosine, but only when IPTG induction was done at room temperature (Figure 3A). No phosphotyrosine containing proteins were detected when induction was done at 37°C . When induction was done at room temperature, the majority of phosphorylated protein was observed in the inclusion body fraction; although phosphorylated protein could also be detected in the soluble fraction (Figure 3B).

It was then asked whether KDR-CD in the soluble fraction of cells has enzymatic activity. KDR-CD was partially purified from the cell supernatant on an anti-phosphotyrosine antibody agarose column and then dephosphorylated

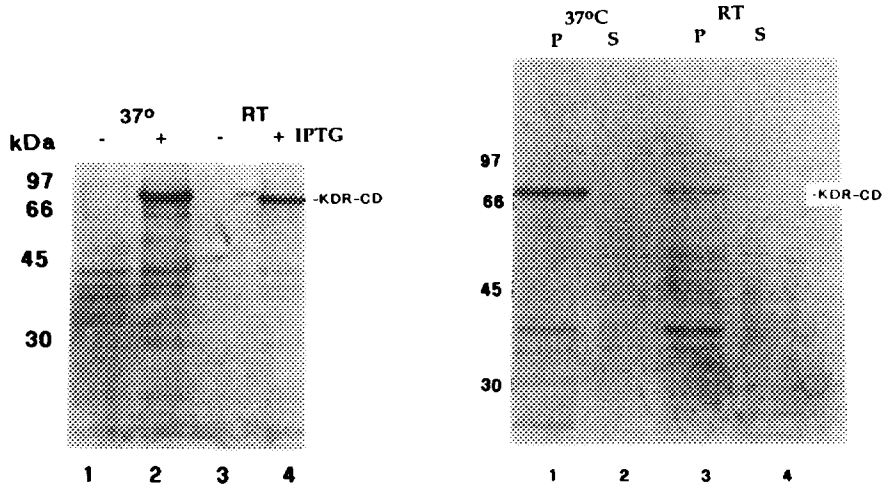

Figure 1. Expression of KDR-CD in *E. coli* BL21. Cells were incubated with (lanes 2 and 4) or without (lanes 1 and 3) IPTG for either 4 hrs at 37°C (lanes 1 and 2) or 16 hrs at 25°C (lanes 3 and 4). Expression of KDR-CD in the cells was analyzed by SDS-PAGE and Coomassie blue staining.

Figure 2. Localization of KDR-CD to the inclusion body fraction: Bacteria were induced with IPTG for either 4 hrs at 37°C (lanes 1 and 2) or 16 hrs at 25°C (lanes 3 and 4). Inclusion body (lanes 1 and 3) and soluble (lane 2 and 4) fractions were isolated. Expression of KDR-CD was analyzed by SDS-PAGE and Coomassie blue staining.

by treatment with acid phosphatase. ATP was added, and KDR-CD autophosphorylation was followed by Western analysis using the anti-phosphotyrosine antibody. Figure 4A shows that phosphatase treatment effectively removed tyrosine phosphates from KDR-CD (compare lane 4 (no phosphatase treatment) with lane 1 (phosphatase treatment)). Addition of ATP to the sample resulted in phosphorylation of the protein (compare lanes 2 (5 minutes incubation with ATP) and 3 (15 minute incubation with ATP) with lane 1 (no incubation with ATP)). This result demonstrates that the bacterially expressed KDR-CD has tyrosine kinase activity. Figure 4B shows that a similar result is obtained if ^{32}P-ATP is added to the dephosphorylated KDR-CD and the kinase activity is analysed by SDS-PAGE autoradiography.

These results suggest that KDR-CD in the soluble fraction possesses tyrosine kinases activity, while KDR-CD in the inclusion body fraction is not enzymatically active. The data are consistent with the notion that soluble KDR-CD uses endogenous ATP to autophosphorylate itself. The presence of phosphorylated KDR-CD in the inclution bodies of cells that are induced at room temperature, but not in cells induced at 37°C may be explained by the kinetics of inclusion body formation as a function of temperature. The lack of

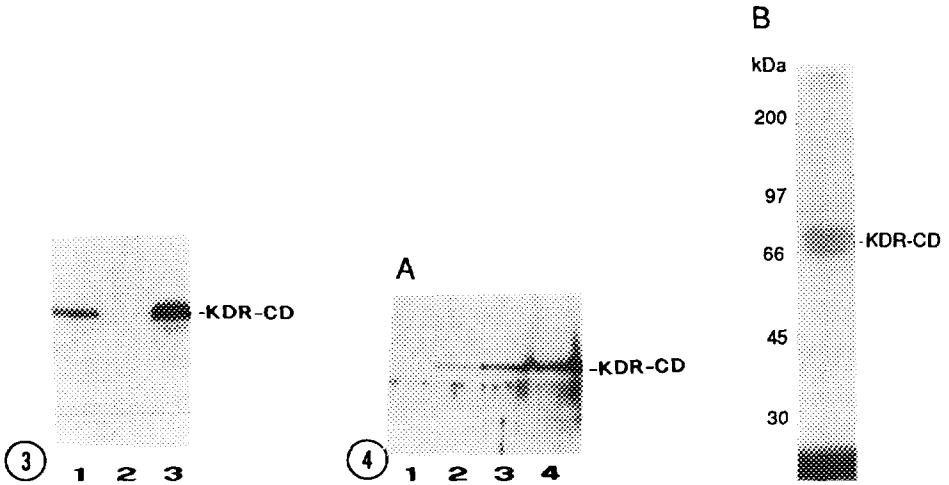

Figure 3. Cellular distribution of tyrosine phosphorylated KDR-CD in bacteria after induction at 25°C. Cells were induced with IPTG for 16 hrs at 25°C and the inclusion body and soluble fractions isolated. The presence of tyrosine phosphorylated KDR-CD in the cellular extract (lane 1), soluble fraction (lane 2) and inclusion body fraction (lane 3) was determined by Western blot hybridization using an anti-phosphotyrosine antibody.

Figure 4. KDR-CD has intrinsic kinase activity. Panel A: KDR-CD, dephosphorylated with acid phosphatase, was incubated with nonradioactive ATP, and the reaction was analyzed by Western blotting. Lane 1 represents affinity-purified KDR after phosphatase treatment, lane 2 is phosphatase-treated KDR-CD incubated with ATP for 5 minutes, lane 3 is phosphatase-treated KDR-CD incubated with ATP for 15 minutes, and lane 4 is KDR-CD not treated with phosphatase. Panel B: The phosphatase-treated KDR-CD was incubated with ^{32}P-ATP for 20 minutes, and the reaction was analyzed by SDS-PAGE autoradiography.

phosphorylated protein in inclusion bodies of cells induced at 37°C may simply be due to the fact that KDR-CD precipitates more rapidly into inclusion bodies at the higher temperature and thus precluding significant autophosphorylation of soluble protein.

In order to determine which of the tyrosine residues of KDR-CD were phosphorylated, the protein was extracted from the inclusion body fraction of bacteria induced at room temperature, solubilized with urea, and purified on an anti-phosphotyrosine antibody affinity column. The phosphorylated KDR-CD was denatured, reduced, carboxyamidomethylated, and then digested with trypsin. The resulting peptides were separated by reverse phase HPLC. A chromatogram from one such experiment is shown in Figure 5A . Several of the resulting fractions contained tyrosine phosphorylated peptides as determined by dot blotting of aliquots of the HPLC fractions and probing with antiphosphotyrosine antibody (Figure 5B). A portion of the tryptic digestion was chromatographed so that late eluting, hydrophobic peptides were resolved

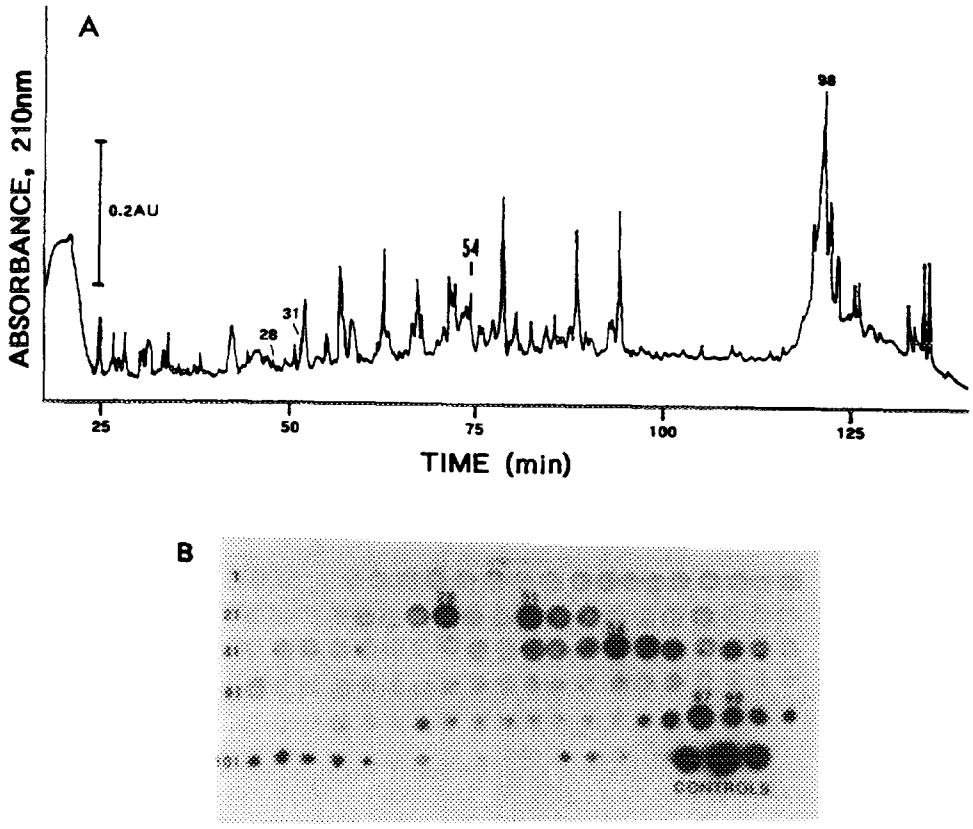

Figure 5. Panel A. Reverse-phase HPLC of phosphotyrosine-containing tryptic peptides from KDR-CD. 100 µg of KDR-CD purified from inclusion bodies was treated with trypsin and the resulting digest separated by reverse phase HPLC. Panel B. Identification of tyrosine phosphate containing peptides. Aliquots of HPLC samples were analyzed for the presence of phosphotyrosine-containing peptides using a dot blot assay. Fraction numbers are indicated. The control samples at the low right of the figure are aliquots of purified KDR-CD before and after protease treatment.

better (data not shown). In addition, several positive fractions from these maps were rechromatographed on shallower gradients and reassayed (also not shown).

N-terminal sequence analysis of phosphorylated peptides is shown in Table 1. Sample 54 contained a single sequence, KDR (945-960), that had a blank cycle where Tyr^{951} was expected (Table 1). The inability to detect Tyr-951 is consistent with it being phosphorylated, since the charged phosphate group prevents extraction of the tyrosyl derivative into the organic phase in the sequencer. While fractions 28 and 31 did not contain single sequences they contained tyrosine phosphate containing peptides KDR(1052-1061) and KDR(1052-1062), respectively. Again blank cycles at either Tyr^{1054} and Tyr

Table 1: Sequence analysis of phosphotyrosine-containing peptides. The fraction numbers at the top of the figure are from the chromatogram shown in Figure 5A. Peptides 28', 31', and 97' were from another chromatogram (not shown) of the same tryptic digest shown in Fig. 5A and correspond to the peaks labeled 28, 31, and 97, respectively. Peptides 28' and 31' were isolated after rechromatography of an unresolved portion of the earlier chromatogram (Conditions: 10% acetonitrile to 25% in 60 min in the presence of 0.1% TFA on the same C18 column used for Fig. 5A). A blank cycle was obtained for those amino acids shown as (-); the residue expected from the KDR sequence is given in parentheses. Yields are calculated from background-subtracted data using ABI data analysis software (Model 610A, version 1.2).

Fraction:	28		28'		31		31'		54		97'	
Cycle	Amino acid	yield (pmol)	Amino acid	yield (pmol)	Amino acid	yield (pmol)	Amino acid	yield (pmol)	Amino acid	yield (pmol)	Amino acid	yield (pmol)
1	D	17.6	D	15.5	D	7.5	D	13.5	F	11.1	S	46.6
2	I	20.5	I	5.0	I	13.3	I	5.6	(R)	-	L	66.5
3	Y	3.2	Y	4.2	(Y)	-	(Y)	-	Q	11.1	S	7.4
4	K	6.1	K	6.5	K	2.3	K	3.2	G	10.2	D	19.6
5	D	8.3	D	2.2	D	5.1	D	1.0	K	11.2	V	17.3
6	P	7.2	P	5.2	P	5.6	P	4.4	D	7.6	E	13.2
7	D	7.2	D	0.5	D	4.8	D	1.3	(Y)	-	E	19.8
8	(Y)	-	(Y)	-	Y	1.2	Y	1.8	V	5.0	E	28.7
9	V	0.5	V	1.5	V	2.3	V	1.8	G	4.5	E	21.3
10	(R)	-	R	0.4	(R)	-	(R)	-	A	8.2	A	26.0
11	K?	1.8	K?	0.7					I	3.4	P	26.5
12									P	3.5	E	14.5
13									V	1.5	D	7.4
14									D	1.3	L	14.7
15									L	3.8	(Y)	-
16									(K)	-	K	11.6
17											(D)	-
18											F	6.1
19											(L)	-
20											T	4.4
21											L	3.4
22											E	4.8
KDR seq	1052-1062		1052-1062		1052-1061		1052-1061		945-960		982-1003	
PO4 site	Tyr^{1059}		Tyr^{1059}		Tyr^{1054}		Tyr^{1054}		Tyr^{951}		Tyr^{996}	

^{1059}, respectively, were observed. These same two peptides were also isolated from rechromatographed maps (data not shown) and the sequencing results are also shown in Table 1 (fractions 28' and 31'). A fraction from another tryptic digestion map (data not shown) that corresponds to peak 97 in Figure 5A contained a fourth phosphotyrosine-containing peptide (97') that is phosphorylated at Tyr^{996}.

DISCUSSION

The finding that KDR-CD has intrinsic tyrosine kinase activity is consistent with previous reports demonstrating similar activity for the cytosolic domains of other receptor tyrosine kinases. The cytosolic domain of the EGF

receptor (EGF-R-CD) (18), when expressed in baculovirus, has intrinsic tyrosine kinase activity. This is also the case for the bacterially expressed cytosolic domain of the FGF-receptor (FGF-R-CD) (19). Furthermore, certain oncogene products (v-erb, v-kit) are receptor tyrosine kinases which lack portions of their ligand binding domains (reviewed in ref. 26). These proteins are constitutively active in the absence of ligand. Of particular note is the v-kit protein which is missing both an extracellular ligand binding and membrane spanning domain.

A variety of evidence suggests that the phosphotyrosines identified using the bacterially expressed KDR-CD correspond to the autophosphorylation sites on the intact receptor after addition of VEGF. First, the autophosphorylation sites on both the baculovirus-derived EGF-R-CD (18) and bacterially-expressed FGF-R-CD (19) are also phosphorylated in their respective intact receptors after addition of growth factor. Secondly, from studies of a number of receptor tyrosine kinases it has been observed that autophosphorylation sites typically contain acidic amino acids one or two residues to the amino terminal side of the phosphorylated tyrosine residues (26). This is the case for each of the four phosphorylation sites mapped on KDR-CD. Thirdly, two of the mapped sites (tyrosines 951 and 996) are contained within the kinase insert domain of KDR. The kinase insert domains of receptor tyrosine kinases are thought to be sites of interaction with cellular signal transduction proteins (22). Tyrosines within the kinase insert domains of other RTKs are known autophosphorylation sites (17), consistent with the hypothesis that this domain defines specificity for cell signaling and activation. Fourthly, two of the mapped sites (tyrosines 1054 and 1059) are contained within a peptide sequence that is conserved in each of the family members (FLT1 (27) and FLT4 (28)) of RTKs to which KDR belongs. The conservation of this sequence suggests a shared signal transducing function of KDR, FLT1, and FLT4.

Several recent studies demonstrate that the amino acid sequences adjacent to autophosphorylation sites define the specificity of receptor interaction with cell signaling proteins containing specific SH2-domains (16,29,30). We have analysed the amino acid sequences surrounding the postulated autophosphorylation sites of KDR for similarity with consensus sequences known to interact with identified SH2 proteins (29). The amino acids sequences adjacent to two of the autophoshorylation sites (Y^{996} and Y^{1054}) show no similarity to any of the reported consensus sequences. The amino acid sequences adjacent to the other two autophosphorylation sites (Y^{951} and Y^{1059}) contain negatively charged amino acids in the -1 position and a valine in the +1 position. This is characteristic of the concensus sequence for interaction of RTKs with several SH2 domains. However, comparison of the amino acids in the +2 and +3 positions of the peptides containing Y^{951} and Y^{1059} with those in corresponding positions of known consensus sequences did not yield

conclusive results, as the amino acids in these positions are not the preferred residues for any known SH2-domain recognition site. It is difficult, therefore, to identify any SH2-domain containing protein which interacts with KDR, as none of the KDR-derived sequences perfectly match the determined consensus sequences. It is important to note, though, that the precise degree of allowed amino acid variation within a given consensus sequence is not well understood.

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