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# The Phosphorylated 1169-Tyrosine Containing Region of Flt-1 Kinase (VEGFR-1) Is a Major Binding Site for PLC$\gamma$

Asako Sawano, Tomoko Takahashi, Sachiko Yamaguchi, and Masabumi Shibuya[1]
*Department of Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108 Japan*

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**Flt-1, a tyrosine kinase receptor for vascular endothelial growth factor (VEGF), plays important roles in the angiogenesis required for embryogenesis and in monocyte/macrophage migration. However, the signal transduction of Flt-1 is poorly understood due to its very weak tyrosine kinase activity. Therefore, we overexpressed Flt-1 in insect cells using the Baculovirus system in order to examine for autophosphorylation sites and association with adapter molecules such as phospholipase C$\gamma$-1 (PLC$\gamma$). Tyr-1169 and Tyr-1213 on Flt-1 were found to be auto-phosphorylated, but only a phenylalanine mutant of Tyr-1169 strongly suppressed its association with PLC$\gamma$. In Flt-1 overexpressing NIH3T3 cells, VEGF induced autophosphorylation of Flt-1, tyrosine-phosphorylation of PLC$\gamma$ and protein kinase C-dependent activation of MAP kinase. These results strongly suggest that Tyr-1169 on Flt-1 is a major binding site for PLC$\gamma$ and important for Flt-1 signal transduction within the cell.** 

A wide variety of tyrosine kinase receptors are known to be intimately involved in cell proliferation, differentiation and morphogenesis in multi-cellular organisms. The seven-immunoglobulin-like domain containing tyrosine kinase receptors Flt-1 and KDR/Flk-1, and their ligand vascular endothelial growth factor (VEGF) have recently been shown to be deeply implicated in both physiological angiogenesis and pathological blood vessel formation such as diabetic retinopathy and solid tumor growth *in vivo* (1-3).

Flt-1 and KDR/Flk-1 are, in most cases, specifically expressed in vascular endothelial cells from the embryogenic to adult stage (4-6). Gene targeting studies clearly showed that these two receptors are essential for angiogenesis. *KDR/flk-1* null mutant mice neither develop endothelial cells nor blood vessels, and were associated with very poor hematopoiesis (7). On the other hand, *flt-1* null mutant mice are embryonic lethal at E8.5 to E9.0 due to blood vessel dysorganization such as overgrowth of endothelial-like cells in the lumen of the vessels (8). These results suggest that Flt-1 induces proper differentiation and morphogenesis of endothelial cells and negatively regulates the proliferation of these vascular cells.

In addition, as an exceptional case, Flt-1 but not KDR/Flk-1, was recently found to be highly expressed on human peripheral blood monocytes and to play an important role in VEGF-dependent cell migration and tissue factor production (9, 10). These functions of monocytes/macrophages may be involved in the progression of inflammation and atherosclerosis *in vivo* (11).

Thus, Flt-1 carries out several important activities in a number of processes ranging from angiogenisis to hematopoietic cell function. However, signal transduction from Flt-1 is still poorly understood mostly as a result of its very low tyrosine kinase activity (12,13). As a limited information, 1213-tyrosine on Flt-1 was recently shown to bind with p85 subunit of phosphatidyl inositol 3-kinase in yeast cell system(14). In order to elucidate the signals elicited by Flt-1 in more details, it seems crucial to establish a system that allows easy detection of autophosphorylation of Flt-1 and identification of its association partners. Using the Baculovirus system and several Flt-1 mutants, we show here that an autophosphorylated tyrosine residue of Flt-1 is involved in its association with PLC$\gamma$, one of the major signal transducers to the cell nucleus.

## MATERIALS AND METHODS

*Cells and antibodies.* Sf9 cells were purchased from Invitrogen (California) and cultured in EX-Cell 400 medium (JRH Biosciences, Kansas). NIH3T3-Flt(1)-3 cells which carry human *flt-1* cDNA in the expression vector BCMGSneo (13) were cultured in 200$\mu$g/ml G418 containing DMEM (Nissui, Tokyo). Antibodies used were anti-Flt-

[1] Corresponding author. Fax: 81-3-5449-5425. E-mail: shibuya@hgc.ims.u-tokyo.ac.jp.



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1(P1-3) rabbit polyclonal antiserum, anti-PLC$\gamma$ monoclonal antibody (mAb) (UBI, New York), anti-phosphotyrosine mAb (PY20) (ICN, Ohio) and anti-active MAPK polyclonal antibody (Ab) (Promega, Wisconsin).

*Site-directed mutagenesis of flt-1.* The ScaI (3619)-XbaI (4262) fragment of *flt-1* cDNA was used for site-directed mutagenesis. The kinase-deficient mutant was designed by substituting lysine-861 in the ATP binding site by methionine (K861M). Three highly conserved tyrosines (Y1169, Y1213, Y1327) were mutated to phenylalanine to generate the mutants Y1169F, Y1213F and Y1327F. Wild type (WT) or mutant forms of *flt-1* cDNA were subcloned into the pVL1393 vector at the EcoRI-NotI site (13) for expression in the Baculovirus system.

*Flt-1 expression using the Baculovirus system.* pVL1393 plasmid DNAs containing various *flt-1* cDNAs were used for the co-transfection of Sf9 cells along with linearized Baculovirus DNA "Baculo Gold" (Phamingen, California). The preparation of recombinant viruses was carried out as described previously (15).

*In vitro kinase assay, phosphopeptide mapping and phosphoamino acid analysis.* Cell lysates derived from Sf9 cells overexpressing wild type or mutant Flt-1s were prepared using 1% TritonX-100 HNTG lysis buffer as described previously (15) and immunoprecipitated with anti-Flt-1 Ab at 4°C for overnight. The immune complexes were collected by precipitation with protein A-sepharose beads (Pharmacia, Sweden). Immunoprecipitates were washed three times with HNTG lysis buffer and two times with 50mM HEPES (PH7.4). Samples were then incubated with kinase buffer [50mM HEPES (PH7.4), 0.1% TritonX-100, 1mM sodium fluoride, 0.1mM sodium orthovanadate, 10mM $MnCl_2$, 2mM $MgCl_2$, 1mM DTT, 0.2% aprotinin, 0.1mM PMSF] containing 10$\mu$Ci of $\gamma$-[$^{32}$P]-ATP (5,000Ci/mmol; Amersham, England) at 30°C for 30min. The kinase reaction was stopped by adding an equal volume of 2 $\times$ sample buffer. The samples were then incubated for 5min at 95°C, spun and resolved on 7% SDS-polyacrylamide gel electrophoresis (PAGE). The radiolabeled proteins were detected by autoradiography. $^{32}$P-labeled Flt-1 proteins were eluted from gel pieces, precipitated with trichloroacetic acid, treated with performic acid and digested with trypsin. Samples were subjected to two-dimensional phosphopeptide mapping or phosphoamino acid analysis on thin-layer cellulose plates by the Hunter thin-layer electrophoresis system (CBS Scientific, California) under standard conditions as described previously (16). Electrophoretic separation in pH1.9 buffer was performed for 25min at 1,000V. Ascending chromatographic separation of the phosphopeptides was performed for 6-7hrs in phosphochromo buffer.

*Analysis of VEGF-dependent tyrosine phosphorylation in NIH3T3-Flt(1)-3 cells.* To detect the autophosphorylation of Flt-1, NIH3T3-Flt(1)-3 cells were grown to 80% confluence, starved in serum-deprived DMEM for 24hrs, and then stimulated with 50ng/ml VEGF for 5min or 60min. GF109203X(GFX) (Sigma, Missouri) was dissolved in dimethylsulfoxide (DMSO). To inhibit protein kinase C (PKC), cells were pre-treated with GFX (5$\mu$M) or DMSO (control) for 60min before stimulation with VEGF. Cell lysates were prepared with 0.5% TritonX-100 HNTG lysis buffer. For immunoprecipitation, cell lysates were incubated with anti-Flt-1 Ab or anti-PLC$\gamma$ mAb at 4°C for overnight. The resulting immune complexes were collected by precipitation with protein-A or -G sepharose beads (Pharmacia, Sweden). Immunoprecipitates were washed three times with 0.5% TritonX-100 HNTG lysis buffer. These samples were separated by 7.5% SDS-PAGE and transferred to nylon membranes. After transfer, the membranes were incubated in blocking buffer at 4°C for overnight. The membranes were then probed with the appropriate antibody, anti-

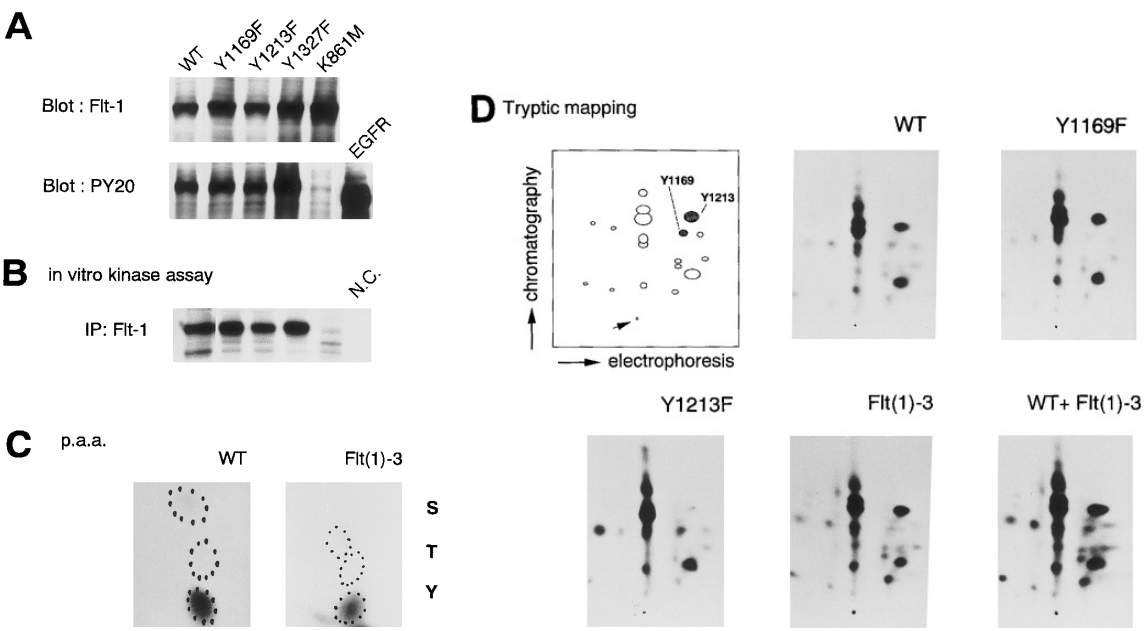


**FIG. 1.** Tyrosine phosphorylation of Flt-1 expressed in insect cells or NIH3T3 cells, phosphoamino acid analysis and tryptic peptide mapping. A: Flt-1 mutants derived from Sf9 insect cells were lysed, separated on SDS-PAGE and blotted with anti-Flt-1 or anti-phosphotyrosine Ab (PY20). B: Immunoprecipitated Flt-1 from cell lysates indicated in A were analyzed by an *in vitro* kinase assay as described in Materials and Methods. N.C.: negative control. C, D: $^{32}$P-labeled wild type or mutant Flt-1s in panel B were used for phosphoamino acid analysis (C) and for tryptic peptide mapping (D). Tryptic peptides were separated by electrophoresis at pH1.9 followed by ascending chromatography. Radiolabeled spots were visualized by autoradiography. The origin is marked with an arrow. The amounts of samples loaded were 500cpm (counts per minute) in (C) and 2000cpm and 4000cpm for separate and mixed samples, respectively, in (D). Flt(1)-3: map of Flt-1 immunoprecipitated from NIH3T3-Flt(1)-3 cells; WT, Y1169F, Y1213F and Y1327F: maps of Flt-1 immunoprecipitated from Baculovirus-infected Sf9 cells.

Flt-1, anti-PLCγ, anti-phosphotyrosine (PY20), or anti-active MAPK Ab, followed by HRP-conjugated secondary antibodies (Amersham, England). Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham, England).

*Interaction of Flt-1 with PLCγ in vitro.* Sf9 cell lysates (200μg protein) containing wild type or mutant Flt-1s were mixed with NIH3T3 cell lysates (500-1000μg protein) and incubated at 4°C for 1hr. The mixture was then used for the immunoprecipitation reaction described above.

## RESULTS AND DISCUSSION

### *Tyr-1169 and Tyr-1213 on Flt-1 Are Autophosphorylation Sites in Insect Cells*

To obtain sufficient amounts of tyrosine-phosphorylated Flt-1 molecules and to identify the autophosphorylation sites important for signal transduction, we constructed several Flt-1 mutants and expressed them in Sf9-insect cells using the Baculovirus system (Fig.1A upper panel). The mutants included a kinase-negative mutant with methionine (K861M) instead of lysine (K) at position 861 and three point mutants Y1169F, Y1213F and Y1327F with phenylalanine (F) instead of tyrosine(Y) at these positions. These three tyrosines are known to be phylogenetically conserved in the carboxyl terminal region of Flt-1 among humans, mice and rats (17-19).

With the exception of the K861M mutant, the wild type and mutants of Flt-1 were highly phosphorylated on their tyrosine residues in insect cells (Fig. 1A lower panel). The kinase-negative K861M mutant did not show any tyrosine phosphorylation, indicating that the phosphorylation of wild type and mutant Flt-1 molecules is due to autophosphorylation by Flt-1, and not by other tyrosine kinases.

The kinase activity of these Flt-1s was constitutively elevated and VEGF-independent. This property resembles that of other receptor-type tyrosine kinases such as PDGFR and EGFR expressed in insect cells (20-22).

The tyrosine kinase activity of these Flt-1 constructs was confirmed by an *in vitro* kinase assay. All constructs except for K861M clearly showed phosphorylation on these molecules (Fig.1B). Further, phosphoamino acid analysis of wild type Flt-1 revealed that the phosphorylation occurred on tyrosine but not on serine or threonine residues (Fig.1C). These results indicate that the Baculovirus-insect cell system is quite useful as a model system to study the phosphorylation sites and signal transduction of Flt-1, whose tyrosine kinase activity is usually weak in a mammalian cell background.

We next searched for the positions of tyrosine autophosphorylation sites on Flt-1 using 2-dimensional tryptic mapping. The pattern of tyrosine-phosphorylated tryptic peptides using Flt-1 expressed in Sf9 insect cells was essentially the same as that of Flt-1 expressed in NIH3T3 cells (see below) (Fig.1D). This result further confirms that the Baculovirus-insect cell system is useful for analyzing the autophosphorylation sites on Flt-1, as was shown previously in the case of FGFR (23). As shown in Fig.1D, the Y1169F and Y1213F mutants of Flt-1 lost their phosphotyrosine spots, indicating that Y1169 and Y1213 are autophosphorylation sites. The Y1327 mutation did not change the number or the intensity of the spots observed in 2-dimensional mapping, suggesting that Y1327 may not be a strong phosphorylation site (data not shown).

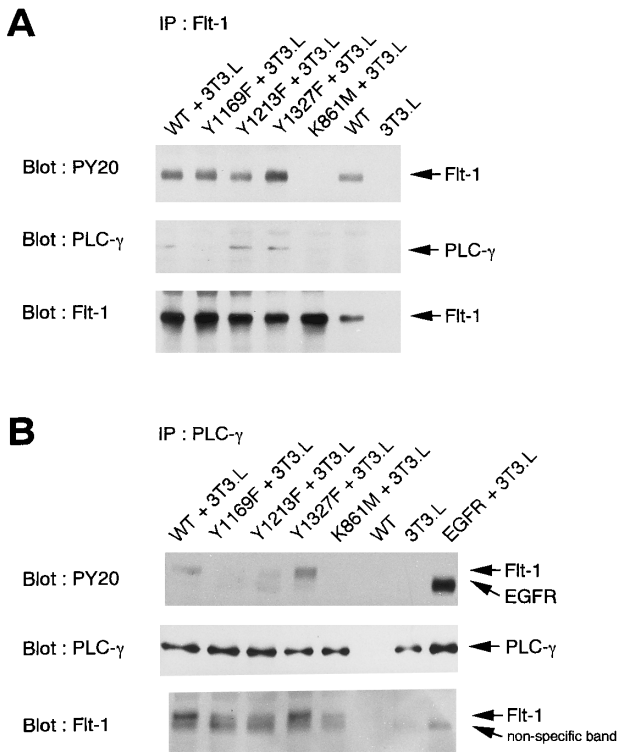


**FIG. 2.** Interaction of wild type or mutant Flt-1 molecules with PLCγ *in vitro.* Sf9 cell lysates expressing the wild type or mutant human Flt-1 molecules were mixed with endogenous PLCγ-containing NIH3T3 cell lysates (3T3.L) and incubated at 4°C for 1hr. The mixture was immunoprecipitated with anti-Flt-1 (A) or anti-PLCγ (B) Ab. After extensive washing, the immune complexes were blotted with anti-phosphotyrosine (PY20), anti-PLCγ or anti-Flt-1 Ab (see Materials and Methods).

This observation is consistent with a recent report by Cunningham et al. where they describe Y1213 as a phosphorylation site (14). In addition, de Vries et al. showed that Flt-1 truncated at residue 1273 was as effective as the wild-type in releasing calcium from Flt-1 expressing Xenopus leavis oocytes in response to VEGF stimulation (24). Thus, Y1327 may not be an important phosphorylation site for signal transduction, at least in the Xenopus oocyte system.

### *Tyr-1169 is a Major Binding Site for PLCγ*

Endothelial cells which express the VEGF receptors Flt-1 and KDR/Flk-1 have been shown to tyrosine-phos-

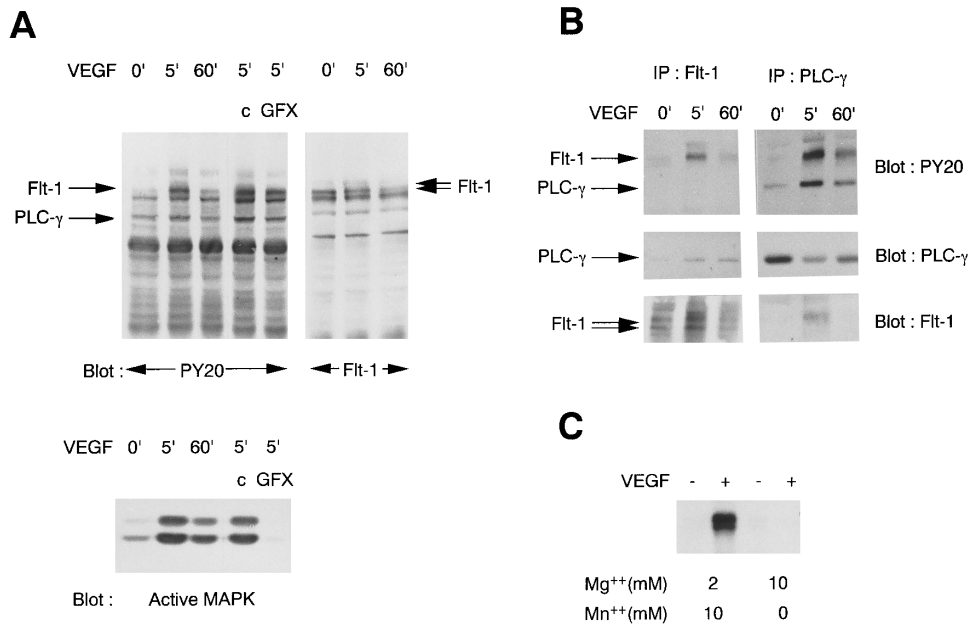


**FIG. 3.** Tyrosine phosphorylation of Flt-1 and PLC$\gamma$ in response to VEGF in NIH3T3-Flt(1)-3 cells. A: NIH3T3-Flt(1)-3 cells were starved overnight in serum-free DMEM and stimulated with VEGF (50ng/ml) for 5min or 60min. In addition, cells were pretreated with DMSO (for control: c) or 5$\mu$M GFX dissolved in DMSO (GFX) for 60min and then stimulated with VEGF for 5min. Cell lysates were separated by SDS-PAGE and transferred to a nylon membrane. Phospho-tyrosine containing proteins were visualized by an anti-phosphotyrosine (PY20) Ab (left). Flt-1 proteins in the same experiment were probed with anti-Flt-1 Ab (right). MAP kinase activities were also detected by anti-active MAPK Ab (lower panel). B: VEGF-dependent PLC$\gamma$ association with Flt-1 and activation of PLC$\gamma$. Aliquots of cell lysates used in A were immunoprecipitated with anti-Flt-1 or anti-PLC$\gamma$ Ab. The immune complexes were blotted with anti-PY20, anti-PLC$\gamma$ or anti-Flt-1 Ab. C: Manganese-dependent Flt-1 kinase activity detected in the *in vitro* kinase assay. Aliquots of VEGF-stimulated or unstimulated NIH3T3-Flt(1)-3 cell lysates were immunoprecipitated with anti-Flt-1 Ab and the immune complexes were incubated with kinase buffer containing $Mg^{++}$ and/or $Mn^{++}$ ions as indicated. Radiolabelled Flt-1 proteins were detected by autoradiography.

phorylate and activate PLC$\gamma$, and to stimulate the downstream PKC pathway in the presence of VEGF (25, 26). Furthermore, Flt-1 has been shown to be expressed on human peripheral blood monocytes and to play an important role in cell migration and tissue factor expression (9, 10). Thus, we examined for possible PLC$\gamma$ binding site(s) by immunoprecipitating mixtures of insect cell lysates expressing the various mutants of Flt-1 and NIH3T3 fibroblast cell lysates containing endogenous PLC$\gamma$.

As shown in Fig. 2A, wild type, Y1213F and Y1327F Flt-1 molecules were specifically bound by PLC$\gamma$ as detected by blotting the anti-Flt-1 immunoprecipitates with anti PLC$\gamma$ mAb. The same results were obtained when anti PLC$\gamma$ immunoprecipitates were blotted with anti Flt-1 Ab or with anti phosphotyrosine mAb (Fig. 2B). As a positive control, the EGF receptor (EGFR) was also tyrosine phosphorylated and bound by PLC$\gamma$ under the same experimental conditions.

However, the association of Y1169F Flt-1 with PLC$\gamma$ was very weak compared to wild type Flt-1 and mutant Flt-1s, Y1213F and Y1327F (Fig.2). Since K861M Flt-1 showed almost undetectable binding of PLC$\gamma$, phosphorylation of the tyrosine residue at position Y1169 must be important for the binding of Flt-1 to PLC$\gamma$. The Y1169-containing amino acid sequence Tyr-Ile-Pro-Ile in humans and the Tyr-Ile-Pro-Leu sequence in mice and rats are homologous to the known PLC$\gamma$ binding sites on PDGFR$\beta$ (1021-Tyr-Ile-Ile-Pro) and on FGFR (766-Tyr-Leu-Asp-Leu). Interestingly, another VEGF receptor, KDR/Flk-1, which is structurally closely related to Flt-1 and capable of activating PLC$\gamma$ in response to VEGF (26), carries the Tyr-Ile-Val-Leu sequence at a similar position at residue 1175. Thus, the Y1169 autophosphorylation site on Flt-1 is most likely a major binding site for PLC$\gamma$.

### *Association of PLC$\gamma$ with Flt-1 and Activation of the PKC Pathway in VEGF-Stimulated Flt-1-NIH3T3 Cells*

In general, the expression of Flt-1 in mammalian cells results in only weak tyrosine phosphorylation (12, 13, 24). To examine the binding of Flt-1 to PLC$\gamma$ and activation of PLC$\gamma$ by VEGF in mammalian cells, we screened more than 40 NIH3T3 cell lines transfected with the Flt-1 expression vector in order to obtain a clone overexpressing Flt-1. In this way we isolated one cell line, Flt(1)-3, that overexpressed Flt-1 in sufficient amounts to allow detection of autophosphorylation and signal transduction.

As shown in Fig.1C and Fig. 3 (A, upper panel, B), stimulation of these cells with VEGF resulted in the rapid but transient autophosphorylation of Flt-1 on tyrosine and association of Flt-1 with tyrosine-phosphorylated PLC$\gamma$. This autophosphorylation of Flt-1 was highly dependent on the presence of $Mn^{++}$ ions, as is the case for most tyrosine kinases (Fig. 3C). In the downstream signaling pathway, MAP kinase was moderately activated, and GFX, a PKC-specific inhibitor, strongly suppressed this MAP kinase activation (Fig.3A lower). These results indicate that Flt-1 stimulates the PLC$\gamma$-PKC pathway to activate the MAP kinase cascade in a manner similar to that of KDR/Flk-1 (26).

When Flt-1 was phosphorylated in an *in vitro* kinase assay in the presence of VEGF, 2-dimensional mapping of the phospho-tyrosine containing tryptic peptides indicated (Fig. 1D) that residues Y1169 and Y1213 of Flt(1)-3 had been phosphorylated.

Taken together, these results indicate that the tyrosine residue located at position 1169 on Flt-1 is the major binding site for PLC$\gamma$ and that this binding serves to activate the PLC$\gamma$-PKC-MAP kinase pathway. Also, our results show that the Baculovirus-insect cell expression system is a useful tool for the analysis of tyrosine kinase signaling when protein kinase activity is limiting.

## ACKNOWLEDGMENTS

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