Idenrification of Vascular Endothelial Growth Factor Receptor-1 Tyrosine Phosphorylation Sites and Binding of SH2 Domain-containing Molecules*

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Nobuyuki Ito‡, Christer Wernstedt§, Ulla Engström§, and Lena Claesson-Welsh‡

From the §Department of Medical Biochemistry and Microbiology, Uppsala University, Biomedical Center, Box 575, S-751 23 Uppsala and the ¶Ludwig Institute for Cancer Research, Uppsala Branch, Biomedical Center, Box 595, S-751 24 Uppsala, Sweden

Receptor tyrosine phosphorylation is crucial for signal transduction by creating high affinity binding sites for Src homology 2 domain-containing molecules. By expressing the intracellular domain of Flt-1/vascular endothelial growth factor receptor-1 in the baculovirus system, we identified two major tyrosine phosphorylation sites at Tyr-1213 and Tyr-1242 and two minor tyrosine phosphorylation sites at Tyr-1327 and Tyr-1333 in this receptor. This pattern of phosphorylation of Flt-1 was also detected in vascular endothelial growth factor-stimulated cells expressing intact Flt-1. In vitro protein binding studies using synthetic peptides and immunoblotting showed that phospholipase C-γ binds to both Y(p)1213 and Y(p)1333, whereas Grb2 and SH2-containing tyrosine protein phosphatase (SHP-2) bind to Y(p)1213, and Nck and Crk bind to Y(p)1333 in a phosphotyrosine-dependent manner. In addition, unidentified proteins with molecular masses around 74 and 27 kDa bound to Y(p)1213 and another of 75 kDa bound to Y(p)1333 in a phosphotyrosine-dependent manner. SHP-2, phospholipase C-γ, and Grb2 could also be shown to bind to the intact Flt-1 intracellular domain. These results indicate that a spectrum of already known as well as novel phosphotyrosine-binding molecules are involved in signal transduction by Flt-1.

Receptor tyrosine kinases comprise a large family of transmembrane receptors for polypeptide growth factors (1). Binding of the growth factor to its specific receptor triggers activation of the intrinsic receptor tyrosine kinase activity. It further provokes autophosphorylation of the receptors and tyrosine phosphophorylation of various intracellular signaling molecules leading to signal transduction to downstream effector molecules (2). Phosphorylation of specific tyrosine residues in the receptors provides high affinity binding sites for a variety of Src homology 2 (SH2) domain-containing proteins (3, 4). The binding of a particular SH2 domain to tyrosine-phosphorylated proteins is dependent on the primary sequence surrounding the phosphotyrosine. Certain SH2 domain-containing proteins such as phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase, and GTPase-activating protein possess enzymatic activities, whereas other SH2 domain molecules, i.e. adaptors like Grb2, Crk, and Nck, lack intrinsic enzymatic activities. Adaptors are believed to transduce signals by mediating protein-protein interactions with other signaling molecules such as the guanine nucleotide exchange factor, Sos (5). Several SH2 domain-containing proteins may converge on the same signal transduction pathway; Grb2, Crk, and Nck has been shown to be involved in Ras activation through binding to the same target Sos. On the other hand, however, it has also been shown that these SH2 domain-containing molecules bind to a variety of other intracellular proteins and seem to be involved in multiple signaling pathways (5).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that promotes endothelial cell proliferation and chemotaxis (6–8) and that modulates the coagulation system by inducing plasminogen activator and plasminogen activator inhibitor (9). The expression of VEGF is induced by hypoxia (10), and VEGF seems to play important roles in many pathological conditions such as tumor vascularization and proliferative retinopathy (11, 12). High affinity receptors for VEGF are expressed predominantly on endothelial cells (13). Two structurally related receptors for VEGF, Flt-1 (VEGFR-1) (14, 15) and KDR/Flk-1 (VEGFR-2) (16, 17), have been identified. They consist of seven immunoglobulin-like loops in the extracellular part, a transmembrane domain, a juxtamembrane domain, a kinase domain interrupted by a 69-amino acid residue long insert, and a C-terminal tail. Recently, several novel VEGF-related polypeptides have been identified and denoted VEGF-B (18), VEGF-C (19), VEGF-D (also known as a fibroblast-stimulating growth factor) (20), and placenta growth factor (21). Although these proteins share about 30–53% homology in their primary sequences, they show distinct patterns of binding to the three known VEGF receptors; VEGF-B and -C bind to KDR/Flk-1, whereas placenta growth factor binds to Flt-1 with lower affinity than VEGF (22). In addition, VEGF-C binds with high affinity to Flt-4 (VEGFR-3) which is expressed on lymphatic endothelium (19).

VEGF receptor expression is seen in various tissues of adult rats, and relatively high expression has been identified during embryogenesis, indicating their very important roles for embryonal development (13). Gene-targeting studies show that both of Flt-1 and KDR/Flk-1 knock-out mice die in utero by blast growth factor receptor; PDGFR, platelet-derived growth factor receptor.

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embryonic day 9.5 (23, 24). Analysis of these knock-out mice revealed an absence of yolk sac-derived blood islands and hematopoietic progenitor cells in KDR/Flik-1 null mice and disorganization of vessels in Flt-1 null mice. These data suggest that the receptors have different biological functions and indicate that Flt-1 and KDR/Flik-1 utilize different signal transduction pathways.

By stimulating KDR/Flik-1-transfected PAE cells and NIH3T3 fibroblasts with VEGF, KDR/Flik-1 has been shown to autophosphorylate (8, 25), and four in vitro tyrosine phosphorylation sites, Tyr-951, Tyr-996, Tyr-1054, and Tyr-1059, have been identified by bacterially expressing the cytosolic domain of the human flt-1 prototype. We aimed to identify the tyrosine phosphorylation sites in Flt-1 and KDR/Flk-1 utilizing different signal transduction molecules involved in Flt-1 signaling. In this report, we aimed to identify the tyrosine phosphorylation sites in Flt-1 by expressing the intracellular (IC) domain of human Flt-1 in the baculovirus system and to identify signal transduction molecules binding to these sites.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Sf9 insect cells (PharMingen) were maintained in Grace's insect cell medium (Sigma) supplemented with fetal bovine serum, yeastolate, yeastolate, and 10% fetal calf serum at 27 °C. An endothelial cell line (MS1; kind gift of J. Arbiser, Department of Surgery, Children's Hospital, Boston) (27) derived from mouse pancreas and immortalized by expression of a temperature-sensitive simian virus large T antigen (HTLE-700; C.B.S. Scientific Co., Inc., Del Mar, CA) according to Boyle et al. (29). First dimension electrophoresis was performed in pH 1.9 buffer (formic acid/glacial acetic acid:double-distilled water, 46:156:179, v/v) for 40 min at 2000 V, and the second dimension ascending gel electrophoresis was run in isoelectric buffer (Tris, glycine, sodium dodecyl sulfate). For two-dimensional phosphoamino acid analysis, the thin layer plates were scraped off and then eluted in pH 1.9 buffer or 30% formic acid and lyophilized. The fractions were subjected to two-dimensional phosphoamino acid analysis and, in parallel, Edman degradation. For Edman degradation, phosphopeptides were coupled to Sequelon-AA membranes (Millipore) according to the manufacturer's instructions and sequenced on an Applied Biosystems Gas Phase Sequencer. The activity in released phenylthiobiotin derivatives from each cycle was quantitated by use of the Bio-Imaging Analyzer.

**Signal Transduction by Flt-1**

**Immunoprecipitation and Immunoblotting**—Insect cells (1 × 10^7) were infected with recombinant baculovirus carrying h-flt-1 IC for 3 days. After washing with ice-cold TBS, cells were lysed with high salt lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 10% glycerol, 2.5 mM EDTA, 100 units/ml aprotinin, 0.1 mM Na3VO4, 2.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). After centrifugation, the supernatants were immunoprecipitated with non-immune serum-coupled agarose gel for 30 min with rocking on ice for 2 h. The immunocomplex was collected with protein A-Sepharose CL-4B, washed with high salt lysis buffer, and resuspended in kinase buffer (20 mM Heps, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.05% Triton X-100, 1 mM dithiothreitol). In vitro phosphorylation was carried out in the presence of [γ-32P]ATP for 30 min at room temperature; the reactions were stopped by addition of 2× sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.1% bromophenol blue, and 2% 2-mercaptoethanol). The boiled samples were electrophoresed on SDS-containing gradient acrylamide gels (7.5–12%) and transferred to nitrocellulose. After exposure to film, bands corresponding to Flt-1 were excised from the filter and digested with trypsin (modified sequencing grade; Promega) or Asp-N (Boehringer Mannheim) for 12 h at 37 °C as described (28). Two-dimensional phosphopeptide mapping was performed using the Hunter thin layer electrophoresis apparatus (Htle-7000; C.B.S. Scientific Co., Inc., Del Mar, CA) according to Boyle et al. (29). First dimension electrophoresis was performed in pH 1.9 buffer (formic acid/glacial acetic acid:double-distilled water, 46:156:179, v/v) for 40 min at 2000 V, and the second dimension ascending gel electrophoresis was run in isoelectric buffer (Tris, glycine, sodium dodecyl sulfate). For two-dimensional phosphoamino acid analysis and, in parallel, Edman degradation, the fractions were subjected to two-dimensional phosphoamino acid analysis and, in parallel, Edman degradation. The sequence of the markers and separated on a cellulose plate at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. After visualization of the markers by ninhydrin (BDH Laboratory Supplies) spraying, the plate was exposed to film.

**Immunobilization of Synthetic Peptides onto Agarose Supports**—The following peptides with or without phosphorylation on tyrosine were synthesized: Ac-KKDKDVDYR1213NYAKFDF (designated as Y1213 without phosphorylation and Y1213Ref with single phosphorylation, Y1213C with double phosphorylation, Y1213 Ref without phosphorylation). The undenatured synthetic peptides were coupled to Sequelon-AA membranes (Millipore) according to the manufacturer's instructions and sequenced on an Applied Biosystems Gas Phase Sequencer. The activity in released phenylthiobiotin derivatives from each cycle was quantitated by use of the Bio-Imaging Analyzer.

**Immunoprecipitation and Immunoblotting**—After incubation with a mixture of [3S]methionine and [35S]cysteine at 100 Ci/ml, the cell lysate was then incubated with immobilized reference sequence KKKPPPDY1242QGDSSTLLA (designated as KKKPPPDY1242QGDSSTLLA and KKKPPPDY1242QGDSSTLLA Ref without phosphorylation). The undenatured synthetic peptides were coupled to Sequelon-AA membranes (Millipore) according to the manufacturer's instructions and sequenced on an Applied Biosystems Gas Phase Sequencer. The activity in released phenylthiobiotin derivatives from each cycle was quantitated by use of the Bio-Imaging Analyzer.

**Protein Binding to Immobilized Synthetic Peptides**—After incubation with a mixture of [3S]methionine and [35S]cysteine at 100 Ci/ml, the cell lysate was then incubated with immobilized reference peptide or phosphorylated peptide in the presence or absence of blocking peptide for 1 h at 4 °C with end-over-end rotation. After washing the gel, binding proteins were separated by SDS-PAGE followed by fixation in destain (7% acetic acid and 10% methanol) for 30 min and incubation in Amplify (Amersham Pharmacia Biotech) for 30 min and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).
pressed in the baculovirus system. Cell lysate and conditioned medium were subjected to two-dimensional phosphopeptide mapping. Fig. 2 shows a two-dimensional phosphopeptide mapping of the Flt-1 IC which was immunoprecipitated with the anti-Flt-1 antibody prior to the immune complex kinase assay. The immunoprecipitated material was separated by thin layer electrophoresis and transferred to a membrane. After exposure to film, the band corresponding to the Flt-1 IC domain was excised, digested with trypsin, and subjected to Edman degradation. Fig. 3 (insets) shows that the peptides from spots a and b contain phosphotyrosine but not phosphoserine nor phosphothreonine. Spot c contains both phosphotyrosine and phosphothreonine, whereas other fainter spots contain phosphoserine or phosphothreonine but not phosphotyrosine. Tryptic peptides eluted from spots a, b, and c were subjected to Edman degradation.

Characterization of Phosphorylated Tyrosine Residues in Flt-1—As shown in Fig. 3, radioactive peaks appeared for spot a at cycle 16, for spot b at cycle 1, and for spot c at cycles 1 and 16. Complete trypsin digestion of the human Flt-1 IC would be expected to give rise to around 50 different peptides. Among these, only two peptides, encompassing Tyr-1242 and Tyr-1333, have a tyrosine residue at position 16. However, the peptide encompassing Tyr-1333 should be negatively charged at pH 1.9 and, therefore, would not migrate to the position of spot a on the two-dimensional mapping. On the other hand, the peptide encompassing Tyr-1242 has +1 charge at pH 1.9, which would be compatible with the position of spot a. In order to confirm this notion, the mutant Y1242F Flt-1 receptor IC domain was expressed in insect cells and subjected to immune complex kinase assays followed by two-dimensional phosphopeptide mapping in the same way as for the wild-type Flt-1 (Figs. 4 and 5). The fact that the spot a in the wild-type receptor analysis was missing from the two-dimensional map of the mutant Y1242F indicates that the peptide encompassing Tyr-1242 matches with spot a, and the Tyr-1242 is one of the tyrosine phosphorylation sites in Flt-1 (Fig. 5).

Similarly, the two tryptic peptides encompassing Tyr-914...
and Tyr-1213 are candidates for the spot \( b \), since only these two peptides have a tyrosine residue at position 1 (Fig. 3B). However, both peptides have +1 charge at pH 1.9 precluding the tentative assignment of one of them as spot \( b \). The two-dimensional mapping of Y1213F and Y1213F mutant receptors show that the spot \( b \) in the wild-type receptor is completely abolished in mutant Y1213F two-dimensional map, whereas it is still present in the two-dimensional mapping of Y914F (Fig. 5, C and D). These results indicate that Tyr-1213 is a second tyrosine phosphorylation site in Flt-1. It is noteworthy that spot \( a \) in the wild-type receptor is missing in the mutant Y914F. Since
Tyr-914 is located in the first kinase domain, it is possible that the mutation of this tyrosine may affect the kinase activity of Flt-1. Accordingly, the overall phosphorylation level of the Y914F mutant IC domain in the immune complex kinase assay was considerably lower than those of the wild-type and other mutant receptors (Fig. 4). We infer from these data that tyrosine phosphorylation of the Flt-1 IC was due to autophosphorylation and not phosphorylation by other kinases present in the Sf9 cells.

The only tryptic peptide matching spot c is that encompassing Tyr-1242, in which Thr-1227 as well as Tyr-1242 are phosphorylated (Fig. 3C). In agreement, spot c as well as spot a were missing from the two-dimensional map of the mutant Y1242F receptor (Fig. 5B).

Thus, Tyr-1213 and Tyr-1242 are the major tyrosine phosphorylation sites in Flt-1. To confirm these results in mammalian cells, porcine aortic endothelial (PAE) cells expressing Flt-1 were stimulated with VEGF (50 ng/ml) subjected to an immune complex kinase assay, and digested with trypsin, followed by two-dimensional mapping (Fig. 6). The two-dimensional mapping revealed spots at positions very similar to those obtained from the Flt-1 IC domain expressed in the baculovirus system (see Fig. 2). Edman degradation confirmed that the spots a and b in Fig. 6 exactly correspond to the peptides encompassing Tyr-1242 and Tyr-1213, respectively (data not shown), indicating that Tyr-1213 and Tyr-1242 are tyrosine phosphorylation sites in Flt-1 expressed in mammalian cells.

**Tyrosine-1213 and Tyrosine-1242 Are Major Phosphorylation Sites in Flt-1**—Most peptide fragments derived from the Flt-1 IC by trypsin digestion will be neutral or positively charged at pH 1.9. However, a peptide encompassing tyrosines 1237 and 1233 (IACCSPPPDYY1237NSVLY1233STPPPI) will be negatively charged at pH 1.9 and would therefore not migrate to the right of the application spot (Δ). To investigate if the peptide YY1327/1333 is tyrosine-phosphorylated, the Flt-1 IC was digested with endopeptidase Asp-N instead of trypsin and subjected to two-dimensional phosphopeptide mapping (Fig. 7). In the wild-type receptor, a few weakly phosphorylated spots (Fig. 7A) as well as strong signals are seen. Phosphoamino acid analysis revealed that spot d contains phosphotyrosine but not phosphoserine and phosphothreonine (Fig. 7B, inset). Edman degradation of peptide material eluted from spot d shows that it contains radioactivity at positions 2 and 8 (Fig. 7B). Among the peptides derived from Asp-N-digested Flt-1 IC, only DY1327NSVLY1333STPPPI contains tyrosine residues at positions 2 and 8. In order to confirm these results, mutant receptors in which Tyr-1327 or Tyr-1333 were substituted with phenylalanine were expressed in insect cells and subjected to two-dimensional phosphopeptide mapping. As a consequence of substituting either of Tyr-1327 or Tyr-1333, spot d disappeared (Fig. 7A), probably by a shift into the middle streak (right side in the figure), which is due to incomplete digestion. These results strongly suggest that both Tyr-1327 and Tyr-1333 are minor phosphorylation sites in Flt-1. In conclusion, we have identified two major tyrosine phosphorylation sites at Tyr-1213 and Tyr-1242 and two minor tyrosine phosphorylation sites at Tyr-1327 and Tyr-1333 in Flt-1.
In Vitro Protein Binding to Synthetic Peptides—We next sought to identify intracellular molecules that may be involved in Flt-1 signal transduction from the identified phosphorylation sites. Phosphorylated or unphosphorylated peptides corresponding to the regions containing Tyr-1213, Tyr-1242, and Tyr-1327/1333 (denoted Y(p)1213 and Y(p)1242 and Y(p)1333Ref, respectively) were immobilized on Affi-Gel matrix, and incubated with 35S-labeled cell lysate derived from metabolically labeled MS1 murine capillary endothelial cells. Several intracellular proteins bound to Affi-Gel-coupled Y(p)1213 and Y(p)1333Ref (Fig. 8). The binding of five proteins with molecular masses around 145, 75, 47, and 25 kDa was competed out by the addition of an excess amount (200 μM) of tyrosine-phosphorylated blocking peptide Y(p)1213 and Y(p)1333 (lane 3), although it was not affected by the addition of an excess amount (200 μM) of reference peptide 1213Ref (lane 3). Similarly, the binding of four proteins with molecular masses 145, 75, 47, and 42 kDa (indicated as a–e, respectively) to the Affi-Gel-coupled Y(pp)1327/1333 was competed out by the addition of an excess amount (200 μM) of tyrosine-phosphorylated blocking peptide Y(pp)1327/1333 (lane 4), although it was not affected by the addition of an excess amount (200 μM) of reference peptide 1333Ref (lane 3).

Identification of SH2 domain-containing proteins that bind to synthetic peptides Y(p)1213 and Y(p)1333—The 145-kDa protein that binds to immobilized Y(p)1213 and Y(p)1333 peptides migrates to the same position as PLC-γ upon SDS-PAGE (data not shown). Immunoblotting with anti-PLC-γ antiserum confirmed binding of PLC-γ to Y(p)1213 and Y(p)1333 but not to 1213Ref and 1333Ref (Fig. 9A). Similarly, the 68- and 25-kDa molecules that bind to Y(p)1213 migrate to the same positions as SH2-containing protein tyrosine phosphatase (SHP-2) and Grb2, respectively (data not shown). Immunoblotting with specific antibodies confirmed that the 68-kDa protein is SHP-2 and the 25-kDa protein is Grb2, respectively (Fig. 9, B and C). The 47- and 42-kDa proteins that bind to Y(p)1333 correspond to the adapter proteins, Nck and Crk, respectively, as shown by immunoblotting (Fig. 9, D and E).

To characterize the 27-kDa molecule that binds to Y(p)1213,
a large scale protein binding experiment was carried out, and the band corresponding to the 27-kDa molecule was excised from preparative SDS-polyacrylamide gels and subjected to protein sequence. As a result, we obtained peptide fragments with sequences homologous to that of Grb2 and the human Grb2-related adaptor protein (Grap) (32), suggesting that a novel Grb2/Grap-like molecule may be involved in Flt-1 signal transduction. Interestingly, the p27-kDa protein is expressed in endothelial cell lines but not in fibroblast and malignant epithelial cell lines (data not shown), suggesting that the molecule may be an endothelial cell-specific signal transduction molecule. The 74- and 75-kDa proteins that bind to Y(p)1213 and Y(p)1333, respectively, remain to be identified.

Association of SH2 Domain-containing Proteins to Immobilized Receptors—In order to show that signal transduction molecules bind to the intact Flt 1 IC domain, and not only to synthetic phosphorylated peptides, we incubated immunoprecipitated baculovirus-derived Flt-1 IC with MS1 murine capillary endothelial cell lysates. Proteins retained by binding to the receptor were visualized by immunoblotting. As shown in Fig. 10A, SHP-2 bound to the wild-type and Y1333F mutant receptor but failed to bind to the Y1213F mutant receptor, in agreement with the results shown in Fig. 9B. This result clearly indicates that SHP-2 associates with Flt-1 and that Tyr-1213 is a specific binding site for SHP-2. On the other hand, Grb2 bound almost as well to the Y1213F mutant receptor as to the wild-type receptor, indicating multiple, probably indirect binding sites for Grb2 to the receptor (Fig. 10B). Similarly, PLC-γ bound to wild-type as well as to the Y1213F and Y1333F mutant receptors (Fig. 10C). Since both Tyr-1213 and Tyr-1333 present binding sites for PLC-γ, it is possible that both sites need to be removed in order to reduce PLC-γ binding.

FIG. 10. Association of SH2 domain-containing proteins to wild-type and mutant Flt-1 receptors. The wild-type and mutant Flt-1 receptor intracellular domains expressed in Sf9 cells were immunoprecipitated with anti-Flt-1 antibody and immobilized on beads. MS1 cell lysate was incubated with receptor-immobilized beads, and bound proteins were separated by SDS-PAGE, followed by immunoblotting using specific antibodies against SHP-2 (A), Grb2 (B), and PLC-γ (C).

FIG. 11. Schematic illustration of the Flt-1 intracellular domain. The different subdomains of the receptor are as indicated: juxta-membrane domain (JM), tyrosine kinase domain 1 (TK1), kinase insert (KI), tyrosine kinase domain 2 (TK2), and C-terminal tail (CT). Open circles indicate position of tyrosine residues, and filled circles indicate positions of tyrosine phosphorylation sites. Binding is shown of the different phosphotyrosine-binding proteins SHP-2, p27, Grb2, PLC-γ, Crk, and Nck to the major (P, phosphate in oval) or minor (P, phosphate in circle) phosphorylation sites.

DISCUSSION

In this paper, we show that Flt-1 is phosphorylated at four positions in the C-terminal tail (Fig. 11). Treatment of Flt-1 expressing cells with VEGF has been shown to only very weakly stimulate tyrosine phosphorylation of this receptor (8, 33). This may be because there are only a few phosphorylation sites in the receptor that may be phosphorylated with low stoichiometry; alternatively, Flt-1 is tightly regulated by phosphatases in mammalian cells. We expressed the Flt-1 intracellular domain in the baculovirus system, to obtain sufficient material for analyzing in vitro phosphorylation of Flt-1 (Fig. 1). It has been shown that the pattern of tyrosine phosphorylation sites in insect cell-derived EGFR and FGFR-1 IC exactly correspond to those identified in intact receptors expressed in mammalian cells (30, 31). Using this strategy, we identified two major tyrosine phosphorylation sites at Tyr-1213 and Tyr-1242, as well as two minor tyrosine phosphorylation sites at Tyr-1327 and Tyr-1333 in Flt-1, and show specific binding of five previously known SH2 domain-containing proteins (Table I) to these sites in vitro. Thus, PLC-γ binds to phosphorylated peptides containing either Y(p)1213 or Y(p)1333, whereas SHP-2 and Grb2 bind to Y(p)1213, and Crk and Nck bind to Y(p)1333. In addition, three unidentified proteins with apparent molecular masses around 75, 74, and 27 kDa bind to Flt-1 in a phosphotyrosine-dependent manner. No significant phosphotyrosine-dependent binding was detected to the peptide containing Y(p)1242. Moreover, phosphorylation of Tyr-1327 appears not to be important for binding of signal transduction molecules, since the pattern of protein binding to Y(p)1213/1327/1333 was essentially identical to that of Y(p)1333. We found no indications for the presence of additional major tyrosine phosphorylation sites, although it is possible that Tyr-794 and Tyr-815, which were not included in the Flt-1 IC domain construct, may be additional Flt-1 phosphorylation sites. Thus, spots not indicated by letters in Fig. 2 were present also in uninfected Sf9 cells and therefore unrelated to Flt-1 (data not shown). Moreover, we show that intact VEGF-stimulated Flt-1 derived from mammalian cells is phosphorylated at the same positions as the baculovirus-derived intracel-
Several receptors such as PDGFR-α and -β and EGFR are equipped with two PLC-γ-binding sites with different affinities (34, 35). In Flt-1, PLC-γ may bind to both pY(1213)VNAFK and pY(1333)STTPPI (Table I). The sequences of the high affinity binding sites for PLC-γ are pYIIPL in PDGFR-α and -β and pY992/LIPQ in EGFR. Thus, phosphorylated Tyr-1333 and its surrounding sequence could provide high affinity binding sites for PLC-γ in vitro (see Table I). The Y1333F mutant receptor still retained the ability to bind PLC-γ, which could be due to the presence of additional binding sites, either at Tyr-1213 or Tyr-1169, as reported by Sawano et al. (36). Sawano and co-workers (36) examined tyrosine phosphorylation of Flt-1 in vivo in Sf9 cells. Phosphopeptide spots on two-dimensional analyses were missing for mutants Y1169F and Y1213F; the Y1169F mutant receptor was unable to bind PLC-γ. Our data agree with the identification of phosphorylation at Tyr-1213, but we could not detect phosphorylation of Tyr-1169.

Grb2, a small adaptor protein carrying one SH2 and two SH3 domains, forms a stable complex with the nucleotide exchange factor Sos which regulates the activity state of Ras. The activated Ras further activates the mitogen-activating protein kinase caspase cascade which is a major mitogenic and motogenic pathway (37). Activation of mitogen-activating protein kinase by VEGF has been shown in Flt-1-transfected fibroblasts and sinusoidal endothelial cells (38) and by placenta growth factor in Flt-1-transfected PAE cells (33). It is quite conceivable that the activated Flt-1 directly or indirectly recruits Grb2, leading to mitogen-activating protein kinase activation in vitro. Indeed, Grb2 binding to the activated Flt-1 can be detected in mammalian cells (data not shown). The small adaptor protein Crk and Nck that may bind to phosphorylated Tyr-1335 in Flt-1 have also been shown to interact with Sos and are implicated in Ras activation (39, 40). Moreover, activation of Jun kinase by v-Crk through the guanine nucleotide exchange protein C3G was recently reported (41). Thus, these adaptor molecules appear to be involved in multiple signaling pathways.

Grb2 may also bind indirectly to Flt-1 via tyrosine-phosphorylated SHP-2, in agreement with previous reports (42). We show the association of SHP-2 with Y(p)1213 in Flt-1 in vitro (Figs. 9B and 10A). The sequence requirements for binding of SHP-2 have been studied with PDGFR-β and insulin receptor substrate-1 (43). It indicated the importance of the presence of β-branched residue such as Val, Ile, and Thr at position pY + 1, Val, or Leu at position pY − 2, and hydrophobic residue with an aliphatic side chain at position pY + 3. Since the sequence around Tyr-1213 has Val at positions +1 and 2, it is conceivable that phosphorylated Tyr-1213 provides a binding site for SHP-2 in vitro (Table I).

**Table I**

| Flt-1 derived sequence | SH2 domain molecule | Assigned binding sequence |
|------------------------|---------------------|--------------------------|
| VYpY(1213)VNAFK         | Grb2                | pYXX(\(a\))              |
|                        | SHP-2               | pY(1213)VP                 |
|                        | PLC-γ              | pYVNL/I/PP                 |
|                        | Crk                 | pYXXX(p)                   |
|                        | Nck                 | pYDTG/P                    |

*Bold letters indicate matching with Flt-1 derived sequences.

The biological function of Flt-1 is poorly understood. Up-regulation of Flt-1 expression has been shown under hypoxic conditions (11, 12), suggesting an important role of this receptor in angiogenesis. The fact that the Flt-1 knock-out mice display disorganization of vessels (23) whereas the KDR/Flk-1 knock-out mice show absence of yolk sac-derived blood islands and hematopoietic progenitor cells (24) indicates distinct mechanisms in the angiogenic process for these two receptors. Whether critical endothelial cell functions such as organization and differentiation of endothelial cells leading to tube formation are guided by signal transduction molecules present only in endothelial cells is an important question. Our peptide binding data indicate that molecules present in endothelial cells but not in a spectrum of other cell types bind to Flt-1-derived sequences in a phosphotyrosine-dependent manner. Our future efforts will be focused on the structural characterization of these molecules.

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