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Tyrosine 1213 of Flt-1 Is a Major Binding Site of Nck and SHP-2

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Vascular endothelial growth factor (VEGF) binds to its receptor tyrosine kinases Flt-1 and KDR/Flk-1 and stimulates their autophosphorylation. However, little is known about their downstream signal transduction properties. We examined the interactions of certain proteins with a SH2-domain with Flt-1 and KDR using the yeast two-hybrid system and found that Nck, SHP-2, PLCγ, and PI3K p85 bind to Flt-1. Extensive site-directed mutagenesis of Flt-1 revealed their major binding sites. Nck, SHP-2, and PI3K bind to Y1213 of Flt-1. Nck also binds to Y1333 of Flt-1. These results suggest that Nck, SHP-2, PLCγ, and PI3K play important roles in Flt-1 signal transduction and that Y1213 of Flt-1 is a major binding site of PI3K, Nck, and SHP-2.

Vascular endothelial growth factor (VEGF) stimulates mitogenicity (1) and chemotaxis (2) in endothelial cells through binding to two different receptor tyrosine kinases, designated Flt-1 and KDR (3). Binding of VEGF to them induces autophosphorylation of their tyrosine residues (2). The phosphorylated receptors are thought to recruit proteins with SH2 domain or phospho-tyrosine-binding domain to their phosphorylated tyrosine residues.

Immunological studies suggested that PI3K p85, PLCγ, Nck and GAP bind to both Flt-1 and KDR in bovine aortic endothelial cells (4). Using the baculovirus expressed Flt-1 as a probe, PLCγ has been shown to bind to the phosphorylated Y1169 of Flt-1 (5). The yeast two hybrid analysis revealed that both N- and C-SH2 domains of PI3K p85 bind to Y1213 of Flt-1 (6) and N- but C-SH2 domain of PLCγ binds to Y794 and Y1169 of Flt-1 and Y801 and Y1175 of KDR (7).

In addition to these proteins, the following reports suggest that another three proteins, Grb2, SHP-2 and Stat3 bind to Flt-1 or KDR.

Grb2 and tyrosine phosphatase SHP-2 have been shown to bind to KDR in KDR-overexpressing porcine aortic endothelial cells in a VEGF-dependent manner (8). Whether they also bind to Flt-1 is currently unknown. Stat3, a transcription factor, has recently been reported to function as an adapter protein. (9). Flt-1 has the consensus motif (YXXQ) for Stat3 binding (10) in its cytoplasmic region (YSFQ; 1002 - 1005 amino acids).

In this paper, we report the yeast two hybrid analysis of the interaction of Nck, Grb2, SHP-2, Stat3 and GAP with Flt-1 or KDR.

MATERIALS AND METHODS

Yeast two hybrid system. The vector plasmids (pGBT9 and pGAD424) and yeast hosts (SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3 :: GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*) and Y190 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2 :: GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3 :: GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*)) were purchased from CLONTECH. Flt-1 and KDR cDNA were kindly provided from Dr. M. Shibuya. The cytoplasmic domain of Flt-1 (781-1338 amino acids) and KDR (788-1354 amino acids) were PCR amplified and subcloned into pGBT9 and used as bait plasmids. Partial cDNAs (containing SH2 region) of PLCγ, PI3K p85, Nck, Grb2, SHP-2, Stat3 and GAP were cloned by PCR from human placenta cDNA library (CLONTECH) and their sequences were confirmed by DNA sequencing. Their SH2 domains were PCR amplified and subcloned into pGAD424 and used as prey plasmids. Primer pairs were: sense 5′-AGTGCGAATTCAATGAGAAGTGGTTCCATGGG-3′, antisense 5′-ATAAGTCGACGCTGTGGGACAGGCTCTGAAAG-3′ for the N-SH2 of PLCγ. sense 5′-AGTGCGAATTCCCACGAGAGCAAAGAGTGGTAC-3′, antisense 5′-ATAAGTCGACGCTCCTCGTTGATGGGATAGCG-3′ for the C-SH2 of PLCγ. sense 5′-GCGAATTCCCTACTACTGTAGCCA-ACAACGGT-3′, antisense 5′-CGGCGTCGACGGTATTTGGATACT-GGATAAAG-3′ for the N-SH2 of PI3K p85. sense 5′-GCGAATTCG-ATGAAGATTTGCCCCATCATGATGAG-3′, antisense 5′-CCAGAG-TCGACTTCATCGCCTCTGCTGTGCATATACTG-3′ for the C-SH2 of PI3K p85. sense 5′-GCGAATTCAGGCCTTCACTCACTGGAA-

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Abbreviations used: Flt-1, fms-like tyrosine kinase-1; KDR, kinase insert domain-containing receptor; PLCγ, phospholipase C gamma; PI3K, phosphatidyl inositol 3-kinase; Grb2, growth factor binding protein 2; SHP-2, src homology 2 phosphatase 2; GAP, GTP-ase activating protein; SH2, src homology domain 2.

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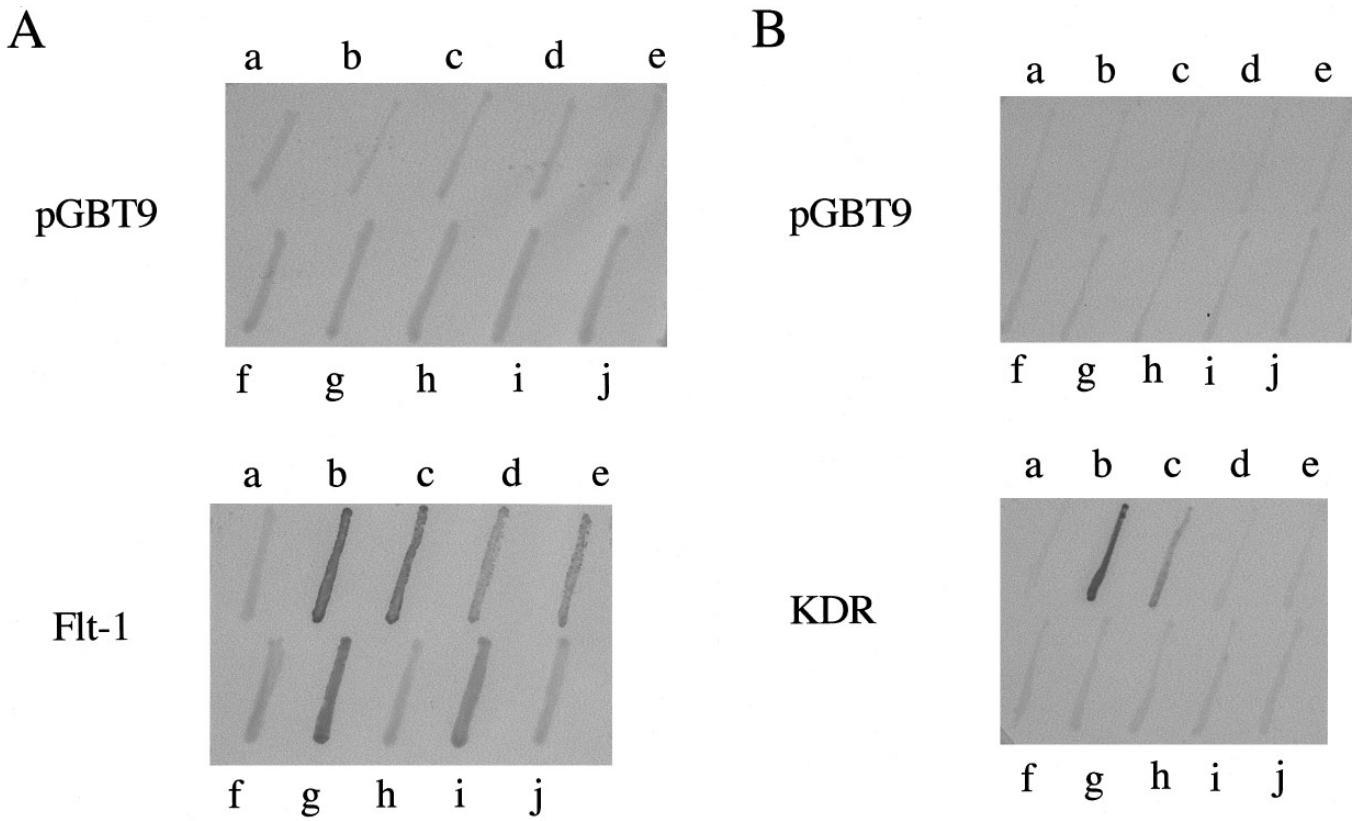

FIG. 1. Interaction of Flt-1 or KDR with certain SH2 domains. The cytoplasmic regions of Flt-1 (A) or KDR (B) were introduced into pGBT9 as baits. The SH2 domains of PLCγ, PI3K p85, Stat3, Nck, Grb2, SHP-2 were introduced into pGAD424 as preys. The resulted bait and prey plasmids were co-transformed into the yeast reporter strains, SFY526 (pGAD424 (a), PLCγ N-SH2 (b), PLCγ C-SH2 (c), PI3K p85 N-SH2 (d) and PI3K p85 C-SH2 (e)) and Y190 (pGAD424 (f), Nck (g), Grb2 (h), SHP-2 N-SH2 (i) and SHP-2 C-SH2 (j)) in various combinations. The transformants were grown on filters and their β-galactosidase activities were visualized using X-Gal as substrate.

AGTTTGC-3′, antisense 5′-CCAGAGTCGACGGTCAGCAGTATC-ATGATAAATGCTTGACAAG-3′ for the SH2 of Nck. sense 5′-CCG-AATTCGTCCTTTTTTGGCAAAATCC-3′, antisense 5′-TACGGTCGA-CTTAGGCTGCTGTGGCACCTGTTC-3′ for the SH2 of Grb2. sense 5′-CCGAATTCATGACATCGCGGAGAT-3′, antisense 5′-TACGGT-CGACTTATGCACAGTTCACACCATATTTAAGC-3′ for the N-SH2 of SHP-2. sense 5′-CCGAATTCTGGTTTCATGGACATCT-3′, antisense 5′-TACGGTCGACTTACTTGAGTTGTAGTACTGTACCC-3′ for the C-SH2 of SHP-2. sense 5′-AGTGCGAATTCATTGGAACCTGGGACC-AAGTGGC-3′, antisense 5′-ATAAGTCGACTCACATGGGGGAGG-TAGCGCAC-3′ for the SH2 of Stat3. sense 5′-TCCCCCGGGGTGGTA-TCACGGAAAACTTGACAGAACG-3′, antisense 5′-TGCGGTCGA-CTCAAACTGGGTAAAGTAATTTTTCTCC-3′ for the N-SH2 of GAP. sense 5′-TCCCCCGGGGTGGTTCCATGGGAAGATTTCC-3′, anti-sense 5′-TGCGGTCGACTCATACAGGTTCCTTAAGATAATATCC-3′ for the C-SH2 of GAP. The resulted bait plasmids and the prey plas-mids were co-transformed into SFY526 or Y190 yeast hosts.

β-Galactosidase assay. The filter assay was performed according to the method of Bartel et al. (11). Briefly, the transformants were streaked directly on a nylon membrane placed on Leu-Trp-His+ plate and incubated at 30°C for a day. The filter was then transferred into liquid nitrogen for 20s and allowed to thaw at room temperature. The filter was placed on a paper filter that was presoaked in Z buffer/X-Gal solution (22.2 g/L $Na_2HPO_4 \cdot 12H_2O$, 5.9 g/L $NaH_2PO_4 \cdot 2H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4 \cdot 7H_2O$, and 390 μg/L X-Gal) and incu-bated at 30°C for about 8 hr. Liquid culture assays using a chemilu-minescent substrate (Galacton Plus (BOEHRINGER MANNHEIM)) for the quantification of β-galactosidase activity were performed ac-cording to the method described in Yeast Protocols Handbook (CLONTECH). Briefly, the transformants were grown in Leu-Trp-His+ medium to saturation overnight and diluted the following morning. Diluted yeasts were grown to an optical density (A600) of about 0.6 and pelleted. Yeasts were washed in Z buffer once and resuspended in 300 μl Z buffer and placed in liquid nitrogen until frozen. Then, yeasts were thawed by incubating in a 37°C water bath for 1 min. This freeze/thaw cycle was repeated once and yeast solu-tion were centrifuged for 5 min. The supernatants of yeast were collected and used as yeast extract. Enzyme reaction and measure-ment of the light reaction were performed according to the manufac-turer's protocol.

Site-directed mutagenesis. Site-directed mutagenesis of Flt-1 was carried out using the Chameleon double-stranded site-directed muta-genesis kit (Stratagene) as described in the manufacturer's instruc-tions. Mutating oligonucleotides were synthesized as follows: Y794F, GAAATAAAGACTGACTTCCTATCAATTATAATGGACCC; Y815F, GTGAGCGGCTCCCTTTTGATGCCAGCAAGTG; K861A, GGACTG-TGGCTGTCGCGATGCTGAAAGAGG; Y990F, TTCTGACGGTTT-CTTCAAGGAGCCC; Y1169F, CAACAGGATGGGAAAGACTTCATC-CCAATC; Y1184F, ATAGTGGGTTTACATTCTCAACTCCTGCCTTC; Y1213F, GGAAGCTCTGATGATGTAAGATTTGTGAATGCTTTC-AAG; Y1242F, CCATGTTTGATGACTTCCAGGGCGACAGC; Y1309F, GCGCAGGTTCACCTTCGACCACGCTGAG; Y1327F, CCGCCCCCA-GACTTCAACTCGGTGGTCC; Y1333F, CTCGGTGGTCCTGTTCTC-CACCCCACCCATC, and to knock out the AatII site as described: TAA-GTAAGTAAGCCGTCGAGCTCTAAGTAAGTAAGC.

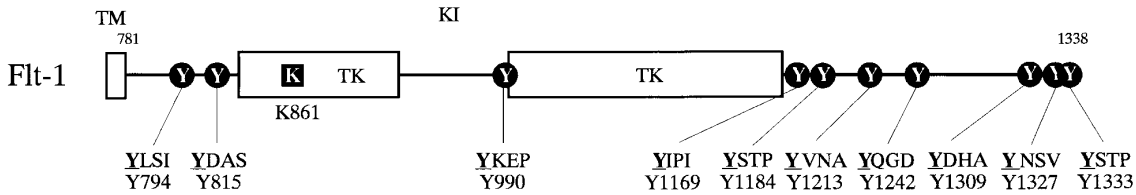

FIG. 2. Schematic of Flt-1 cytoplasmic region. Figure points out mutated tyrosine residues in this study. TM, transmembrane; KI, kinase insert; TK, tyrosine kinase.

RESULTS AND DISCUSSION

Nck and SHP-2 bind to Flt-1. In order to examine the binding of Flt-1 or KDR to SH2 domains of Nck, SHP-2 and other proteins, we co-expressed the cytoplasmic region of Flt-1 or KDR with each SH2 domain of them in the yeast two hybrid system. In this system, reconstitution of functional transcription factor through the binding between a bait protein and a prey protein leads to the expression of the reporter gene b-galactosidase. The cytoplasmic region of Flt-1 or KDR was introduced into pGBT9 as a bait. The SH2 domains of PLCγ, PI3K p85, Nck, Grb2, SHP-2, Stat3 and GAP were introduced into pGAD424 as preys. The resulted bait and prey

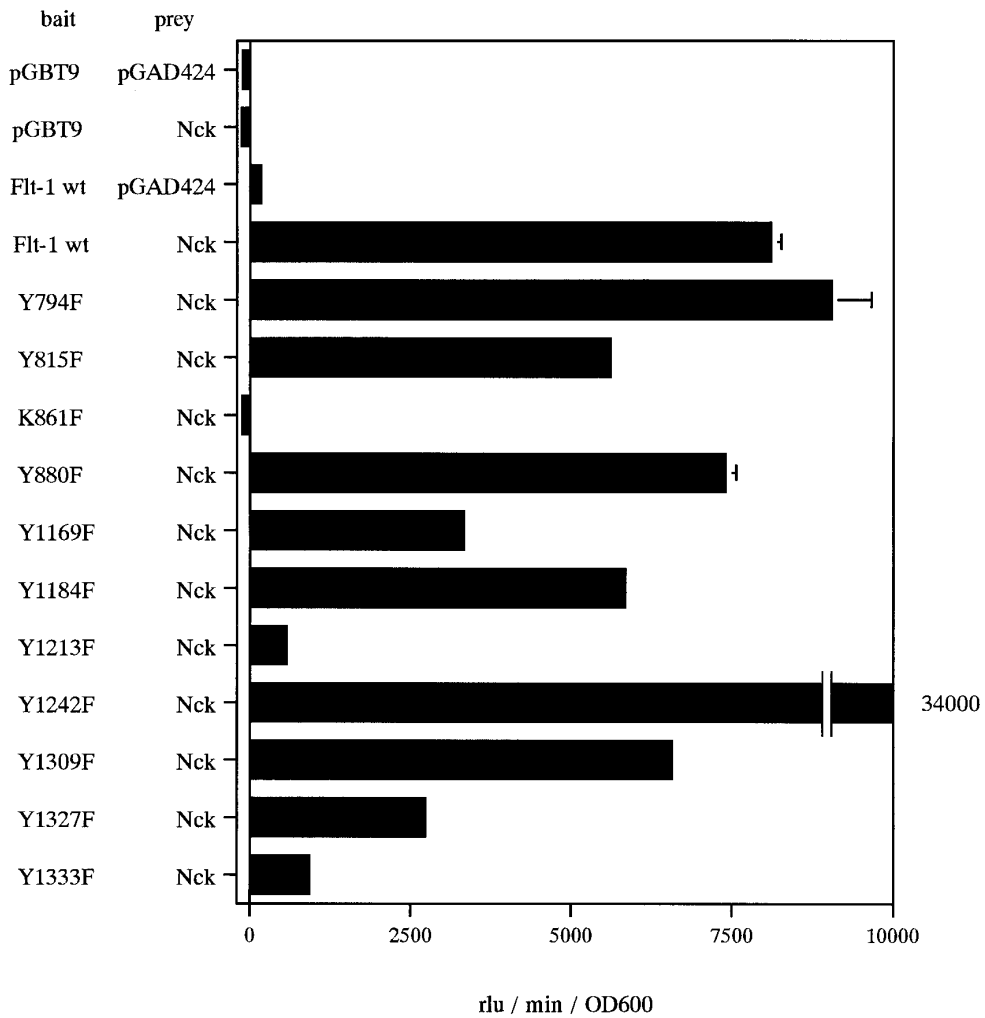

FIG. 3. Interactions of Nck SH2 with Flt-1 cytoplasmic region mutant constructs. Liquid assay of β-galactosidase activities expressed in yeast host Y190 using chemiluminescent Galacton Star as substrate. Values represent the mean $\pm$SD of triplicate determinations expressed in relative light units divided by reaction time (60 min) and optical density.

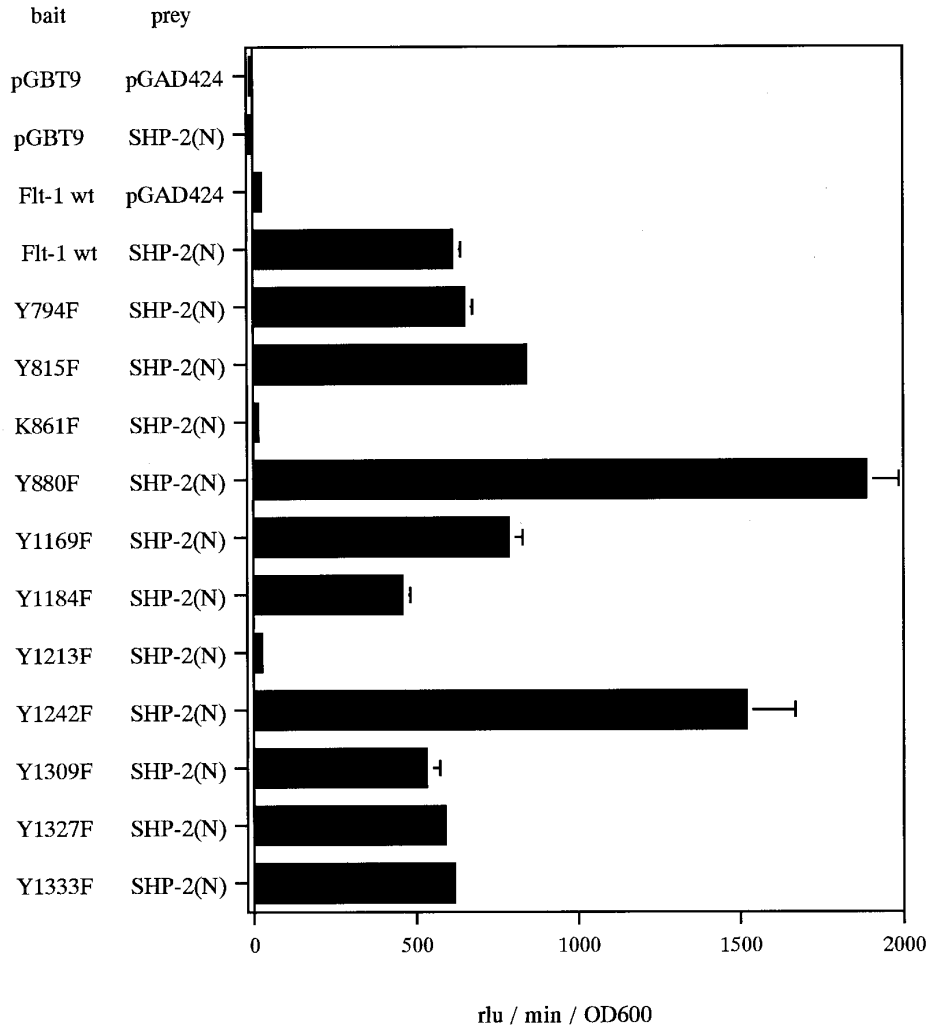

FIG. 4. Interactions of SHP-2 N-SH2 with Flt-1 cytoplasmic region mutant constructs. Liquid assay of β-galactosidase activities expressed in yeast host Y190 using chemiluminescent Galacton Star as substrate. Values represent the mean ±SD of triplicate determinations expressed in relative light units divided by reaction time (60 min) and optical density.

plasmids were co-transformed into the yeast reporter strains, SFY526 and Y190 in various combinations. The transformants were grown on filters and their b-galactosidase activities were visualized using X-Gal as substrate. As shown in Fig. 1A, the N- and C-SH2 domains of PLCγ, the N- and C-SH2 domains of PI3K p85, the SH2 domain of Nck, and the N-SH2 domain of SHP-2 bound to Flt-1. The C-SH2 domain of SHP-2, the SH2 domain of Grb2 did not bind to Flt-1. Further, we could not detect the binding of SH2 domain of Stat3 and GAP to Flt-1 (data not shown).

The N-SH2 of PLCγ bound to KDR as previously reported (7) and the C-SH2 domain of PLCγ also bound to KDR (Fig. 1B). Unexpectedly, we could not detect the significant binding of KDR to the SH2 domain of Grb2 nor both SH2 domains of SHP-2, although the interaction of Grb2 and SHP-2 with KDR has been suggested (8). This discrepancy might be derived from the weak binding of KDR to prey proteins with SH2 domain in the yeast two hybrid system used in this study. Actually, previous report showed that the magnitude of the binding between KDR and N-SH2 domain of PLCγ is relatively weak in comparison to Flt-1 (7). Further, we could not detect the binding of SH2 domain of Stat3 nor GAP to KDR (data not shown). Taken together, these results indicate that Nck and SHP-2 may directly bind to Flt-1.

Nck binds to Y1213 and Y1333 of Flt-1. To determine the tyrosine residues of Flt-1 responsible for Nck binding, we co-transformed the SH2 domain of Nck and Flt-1 mutant constructs whose tyrosine residues were individually mutated to phenylalanine (Fig. 2). b-galactosidase activity of each transformant was quantified using Galacton-Star as substrate. As shown in Fig. 3, the mutation of kinase domain (K861F) causes com-

plete loss of the activity. Substitution of Y1213 resulted in 93% lowering of b-galactosidase activity, and substitution of Y1333 resulted in 89% lowering. Substitution of Y1242 resulted in 321% highering for uncertain reason. These results indicate that two tyrosine residues of Flt-1, Y1213 and Y1333, are responsible for the binding to the SH2 domain of Nck.

Y1213 is followed by VNA, and Y1333 is followed by STP. They do not agree with Y-D-E-P/D/V, the preferred sequence for Nck SH2 domain binding determined from in vitro phosphopeptide studies (12).

SHP-2 binds to Y1213 of Flt-1. Next, we attempted to determine the residues of Flt-1 responsible for SHP-2 binding. We co-transformed Flt-1 mutants described above and the N-SH2 domain or N- and C-SH2 domain of SHP-2. b-galactosidase activity of each transformant was shown in Fig. 4 and the result showed that Y1213 of Flt-1 may be the major binding site for SHP-2. Substitution of Y1213 resulted in 95% lowering of b-galactosidase activity. Y1213 is followed by VNA. As the preferred sequence of SHP-2 N-SH2 domain binding is Y-V/I/T-X-V/L/I (12), Y1213VNA almost matched with the sequence.

As determined previously (6), Y1213 of Flt-1 is responsible for PI3-kinase p85 binding (data not shown). We found that Y1169 of Flt-1 is also responsible (data not shown).

In summary, using the yeast two hybrid system, we showed that Nck may bind to Y1213 and Y1333 of Flt-1 and that SHP-2 may bind to Y1213 of Flt-1. Considering that Y1213 of Flt-1 has been shown to be a binding site for PI3K p85 (6) and is autophosphorylated when Flt-1 was expressed in both yeast and insect cell (5,6), Y1213 of Flt-1 may be autophosphorylated upon the binding of VEGF and become a target for certain SH2-containing proteins, for example PI3K p85, Nck and SHP-2.

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