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Sck Interacts with KDR and Flt-1 via Its SH2 Domain

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Vascular endothelial growth factor (VEGF) is one of the major angiogenesis regulators. It binds to its tyrosine kinase receptors, KDR and Flt-1. However, little is known about their downstream signal transduction properties. We screened human brain cDNA library using the yeast two-hybrid system with the KDR cytoplasmic region as bait to find KDR binding proteins. After 6.2×10^6 clones were screened, we identified Sck, one of the Shc homologues, as a KDR binding protein. Sck also binds to Flt-1 and their binding is dependent on the kinase activities of KDR and Flt-1. Extensive site-directed mutagenesis of KDR revealed that Y1175 of KDR is a major binding site for Sck. As Sck contains the SH2 domain and PTB domain, we tested whether they bind to KDR and Flt-1. The SH2 domain of Sck binds to both of them. Deletion of the SH2 domain from Sck resulted in the complete loss of binding. On the other hand, the PTB domain of Sck does not bind to KDR and Flt-1. These results indicate that Sck binds to KDR and Flt-1 via its SH2 domain and might play an important role in VEGF signal transduction. © 1998 Academic Press

Vascular endothelial growth factor (VEGF), one of the major angiogenic factors, has two endothelial specific receptor tyrosine kinases, designated KDR and Flt-1 (1). It stimulates various responses in endothelial cells, for example, mitogenesis (2), chemotaxis (3), gene expressions [e.g., heparin-binding epidermal growth factor (4), COX-1 (5, 6) and the alpha1 and alpha2 subunits of integrins (7)], reorganization of actin stress fiber, activation of FAK (8), disorganization of junctional proteins (9) and VE-cadherin tyrosine phosphorylation (10). Various intracellular signals corresponding to these cellular responses are initiated from KDR and Flt-1, however, the understanding of these signals are not enough. Immunological studies suggested that PI3K p85, PLCγ, Nck and GAP bind to both KDR and Flt-1 in bovine aortic endothelial cells upon VEGF stimulation (11). Using the baculovirus expressed Flt-1 as a probe, PLCγ has been shown to bind to the phosphorylated Y1169 of Flt-1 (12). The yeast two hybrid analysis revealed that both N- and C-SH2 domains of PI3K p85 bind to Y1213 of Flt-1 (13) and N- but C-SH2 domain of PLCγ binds to Y794 and Y1169 of Flt-1 and Y801 and Y1175 of KDR (14). Shc, Grb2 and tyrosine phosphatase SHP-2 have been shown to form a complex with KDR in KDR-overexpressing porcine aortic endothelial cells in a VEGF-dependent manner (15). Recently, we reported that Nck binds to Y1213 and Y1333 of Flt-1 and SHP-2 binds to Y1213 of Flt-1 (16).

To find KDR binding proteins, we screened human brain cDNA library using the yeast two hybrid system with KDR cytoplasmic domain as a bait. In this paper, we report that Sck, one of Shc homologues, binds to both KDR and Flt-1 in their tyrosine kinase dependent manner.

MATERIALS AND METHODS

Yeast two hybrid system screen. Yeast two-hybrid plasmid pGBT9 containing the cytoplasmic region of KDR (790-1356 amino acids) (KDR/pGBT9) (16)was used as a bait. A human brain cDNA library constructed in prey plasmid pACT2 pretransformed into the yeast strain Y187 (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ*, gal80*Δ*, met*$^-$*, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ)* was purchased from CLONTECH. The screening was performed as described in the manufacturer's instructions. Briefly, the yeast PJ69-2A (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2*) transformed with KDR/pGBT9 was mated with the yeast Y187 pretransformed with the library. The resulted diploids were selected as the colonies growing without adenine and histidine. The β-galactosidase activities of these colonies were assayed and the colonies showing the positive activities were selected. The library plasmids were isolated from the selected colonies and re-introduced into the yeast Y187 to confirm the interac-

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Abbreviations used: Sck, Shc-like; KDR, kinase insert domain-containing receptor; Flt-1, fms-like tyrosine kinase-1; SH2, src homology domain 2; PTB, phosphotyrosine-binding domain; CH, collagen homology domain; 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; PAEC, porcine aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; EGF, epidermal growth factor; EGFR epidermal growth factor receptor; MAPK, mitogen activated protein kinase.

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tion with KDR cytoplasmic region. The positive clone cDNAs were partially sequenced.

Construction of Sck mutants. Partial Sck cDNA was isolated by using the Rapid-Screen cDNA Library Panel human fetal brain (OriGene). Briefly, master 96 well plate was PCR-screened with the primer pair, Sck/SH2/FW (TTCCCTTGGAGGACCACTGG) and Sck/SH2/RV (AGGCGAAATGGTTCCGTCTTTGG). Nine subplates corresponding to the wells showing the positive amplification were re-PCR-screened with the same primer pair. The *E. coli* cells in the positive wells were isolated and PCR-screened with the same primer pair. The resulted candidate clones were sequenced and the clone (8B6A5) containing the PTB domain was isolated.

The Sck constructs used here were PCR amplified and subcloned into the prey vector pACT2. Primer pairs were: sense 5′-GGAATTCGATTCATCCGGAAGGGCAGCTTCATCC-3′, antisense 5′-CCGCTCGAGTCAGGGCTCCCGTGAGACCACG-3′ for the plasmid containing PTB, CH and SH2 domains (Sck PTB-CH-SH2). sense 5′-GGAATTCGATTCATCCGGAAGGGCAGCTTCATCC-3′, antisense 5′-CCGCTCGAGCGGGATGCTGTTGTAGTAATTGTG-3′ for the plasmid containing PTB domain (Sck PTB). sense 5′-GAAGATCTTTCCCTTGGAGGACCAGTGG-3′, antisense 5′-CCGCTCGAGGCGAAATGGTTCCGTCTTTGG-3′ for the plasmid containing SH2 domain (Sck SH2). For the plasmid containing PTB and CH but SH2 domains (Sck PTB-CH), the plasmid Sck PTB-CH-SH2 was digested with *Pvu*II and *Xho*I. The fragment containing the vector sequence was blunt ended and ligated.

Construction of Flt-1 and KDR mutants. Site-directed mutagenesis of Flt-1 and the primers used were described previously (16). The triple mutant of Flt-1 (Y1309F, Y1327F and Y1333F) were constructed using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) with three primers, Y1309F, Y1327F, and Y1333F, as described in the manufacturer's instructions. Site-directed mutagenesis of KDR was carried out using the same kit. The sequences of mutating primers were as follows: Y801F, AACTGAAGACAGGCTTCTTGTCCATCGTCATGG; Y822F, GTGAACGACTGCCTTTTGATGCCAGCAA; K868A, GGACAGTAGCAGTCGCAATGTTGAAAGAAGG; Y951F, AGGGAAAGACTTCGTTGGAGCAATCC; Y996F, CCTGAAGATCTGTTTAAGGACTTCCTGACC; Y1175F, GGATGGCAAAGACTTCATTGTTCTTCCG; Y1319F, CAGACACCACCGTGTTCTCCAGTGAGGAAGC.

β-Galactosidase assay. The filter assay was performed according to the method of Bartel *et al.* (17) and described previously (16).

RT-PCR analysis. cells (5×10^5) of HUVECs [primary culture, passage number 3 (KURABOU)] grown in HuMedia-EG2 (KURABOU) were used as the origin of mRNAs. mRNAs were purified using the mRNA capture kit (ROCHE MOLECULAR BIOCHEMICALS). RT-PCR was performed using Titan one tube RT-PCR system (ROCHE MOLECULAR BIOCHEMICALS) with either the Sck specific primer pair, Sck/P/F4 (AGAGCAACCTTCGCTTTGCC) and Sck/P/B9 (AGGGTCGCATGTCAAACAGATC) or the human β-actin specific primer pair, β-AC/FW (CCAAGGCCAACCGCGAGAAGATGAC) and β-AC/RV (AGGGTACATGGTGGTGCCGCCAGAC). All procedures were followed as described in the manufacturer's instructions.

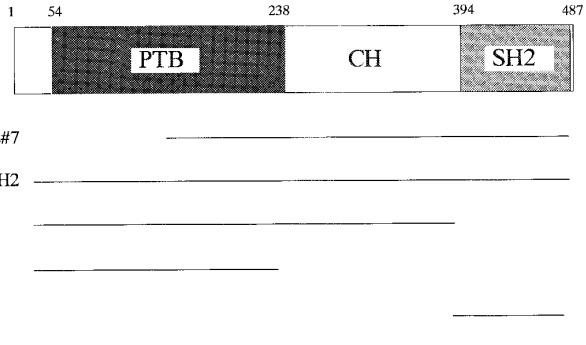

FIG. 1. Structural representation of wild type and mutant Sck. The PTB and SH2 domains are indicated by shaded boxes. The segments of the clones analyzed in this study are indicated by bars.

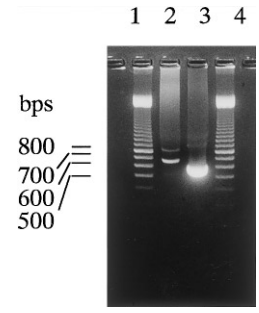

FIG. 2. Expression of Sck mRNA in HUVEC. mRNA purified from 5×10^5 HUVECs grown in growth media were subjected to the RT-PCR using the primer pairs for Sck (lane 2) and β-actin (lane 3). One hundred base pair DNA size markers were loaded at both sides (lanes 1 and 4). Similar results were obtained in three different experiments.

RESULTS AND DISCUSSION

Identification of Sck as a binding protein of KDR. We screened human brain cDNA library using the yeast two hybrid system with KDR cytoplasmic region as a bait. As a screening method, we utilized the selection of the diploids growing without adenine and histidine resulted from the mating the yeast PJ69-2A expressing KDR with the yeast Y187 expressing the library. The yeast PJ69-2A has the two reporter genes, *ADE2* and *HIS3*, therefore, the yeasts having a cDNA for KDR binding proteins are able to grow without adenine and histidine. As a result of two rounds of screening, we obtained 50 colonies growing without adenine and histidine among calculated 6.2×10^6 diploids. In order to remove false positives, we assayed the β-galactosidase activity of these 50 colonies with the filter assay and selected 48 colonies showing the positive β-galactosidase activity. After isolating plasmids derived from cDNA library from these yeasts, we co-transformed these plasmids and KDR/pGBT9 into the yeast Y187. We assayed the β-galactosidase activity of the transformants and selected 38 plasmid clones. Finally, in order to remove the clones binding to the DNA binding region of Gal4 protein, we co-transformed these 38 plasmids and pGBT9 into the yeast Y187 and assayed the β-galactosidase activity of the transformants, but none of them showed the positive β-galactosidase activity. Therefore, we selected these 38 plasmid clones as candidates for cDNAs of KDR binding proteins. We determined the partial DNA sequences of them and searched for the cDNAs having homology to them. Among these, we found the fragment of Sck gene (clone number 2#7), one of Shc ho-

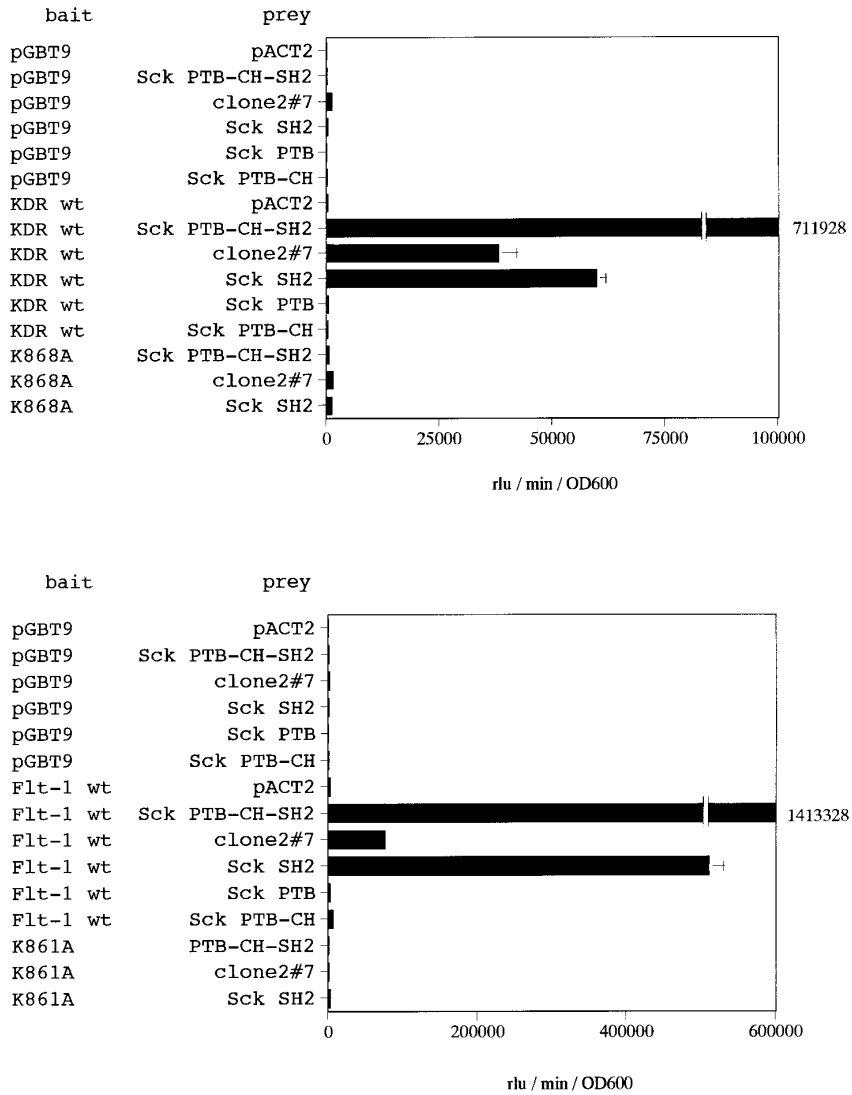

FIG. 3. Sck binds to KDR and Flt-1 via its SH2 domain. The baits are the cytoplasmic regions of wild-type and kinase mutant of KDR (A) or Flt-1 (B) introduced into pGBT9. The preys were the mutants of Sck introduced into pACT2. The bait and prey plasmids were cotransformed into the yeast reporter strain Y187. Liquid assay of β-galactosidase activities of the transformants were assayed using chemiluminescent Galacton Star as a substrate. Values represent the mean $\pm$ SD of triplicate determinations expressed in relative light units divided by reaction time (60 min) and optical density.

mologues. This fragment contained from 616 to 2356 of the partial sequence of Sck described by Nakamura *et al.* (18) (Fig. 1). The analysis of PAEC overexpressing KDR revealed that Shc forms a complex with KDR in a VEGF stimulation dependent manner and becomes phosphorylated on its tyrosine residues (19). These data suggest that Sck might also play in KDR signal transduction in endothelial cells through the binding to KDR. Therefore, we picked up Sck as worth analyzing in detail.

Among other clones, there are 12 clones (clone number 1#7, 1#10, 1#11, 1#13, 1#35, 2#1, 2#2, 2#8, 2#10, 2#13, 2#14, 2#19) that are highly homologous to PI3K p85. Previously, we could not detect the binding of the N- and C-SH2 domains of PI3K p85 to KDR by the yeast two hybrid analysis (16). This discrepancy might be due to the difference of the expression level of PI3K p85. We used the low-level expression vector pGAD424 in the previous report, on the other hand, we used the high-level expression vector pACT2 in the screening. PI3K p85 has been shown to bind to Flt-1 cytoplasmic region by the yeast two hybrid analysis (13). It is also shown that PI3K p85 forms a complex with KDR and Flt-1 in BAEC upon VEGF stimulation (11). The result that PI3K p85 was selected through the screening with KDR cytoplasmic region as a bait suggests that PI3K p85 might play some roles in VEGF signal transduction through the binding to KDR as well as Flt-1. However, it is also shown that VEGF stimulation in endothelial cells failed to activate the PI3K enzymatic

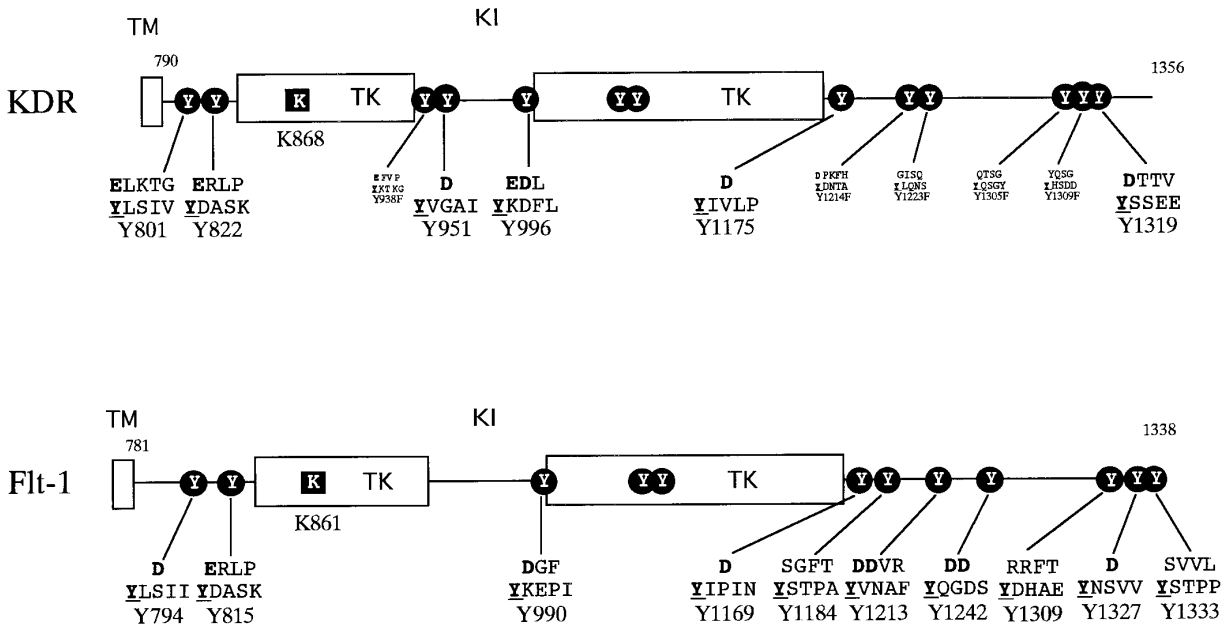

FIG. 4. Schematics of KDR and Flt-1 cytoplasmic regions. Figure points out mutated tyrosine residues in this study (upper: KDR, lower: Flt-1). TM; transmembrane, KI; kinase insert, TK; tyrosine kinase.

activity (8). Therefore, further analysis will be required to reveal the roles of PI3K p85 in VEGF signal transduction. The remaining 25 clones are now under investigation.

Expression of Sck in HUVEC. If Sck plays some roles in VEGF signal transduction, the expression of Sck in endothelial cells must be confirmed. It has been shown that Sck is expressed mainly in human liver, pancreas and prostate (18). However, the expression of Sck in endothelial cells has not been confirmed so far. Therefore, we performed RT-PCR analysis with the specific primers to Sck (corresponding to 509–1189 of AB001451) to confirm the expression of Sck mRNA in endothelial cells. We purified mRNA from human umbilical vein endothelial cell (HUVEC) and used it for the template. The result shown in Fig. 2 revealed the band with the expected length, i.e., 681 bp, was amplified with the Sck specific primer. This result indicates that Sck is indeed expressed in endothelial cells.

Sck interacts with both KDR and Flt-1 and their interaction is kinase-dependent. We tested whether Sck binds to KDR and Flt-1. Using the plasmid Flt-1/pGBT9 to express Flt-1 cytoplasmic region as a bait, we assayed the β-galactosidase activity of each transformant. As shown in Fig. 3, Sck bound to Flt-1 as well as to KDR. Next, we examined whether these bindings are dependent on the tyrosine kinase activities of KDR and Flt-1 using the mutants lost their kinase activities. As shown in Fig. 3, the binding of them was completely dependent on their kinase activities. These results suggest that Sck binds to the phosphorylated tyrosine residues of KDR and Flt-1 upon the activation of KDR and Flt-1 in endothelial cells.

Essential domain of Sck for the binding to KDR and Flt-1 is its SH2 domain but not its PTB domain. There are three domains in Sck protein, i.e., PTB domain (amino acid number 54–238), CH domain (239–393) and SH2 domain (394–487) (18). Among these three domains, PTB domain and SH2 domain might be responsible for the binding to the phosphorylated tyrosine residues of KDR and Flt-1. The partial clone of Sck obtained by the screening has C terminal half of PTB domain and full SH2 domain. This suggests that SH2 domain binds to KDR and Flt-1. In order to construct the prey plasmid expressing various region of Sck, we obtained a cDNA of Sck containing the full length of PTB domain (193–2356) from human brain cDNA library and used it as a template. We first investigated whether SH2 domain of Sck can bind to KDR and Flt-1. β-galactosidase activity of each transformant was quantified using Galacton-Star as a substrate. As shown in Fig. 4, SH2 domain can certainly bind to both of them. Next, we examined whether PTB domain van bind to KDR and Flt-1. As a result, PTB domain could not bind to both of them. In addition, the clone containing full PTB domain and SH2 domain binds to both of them and the clone containing full PTB domain but not SH2 domain could not bind to them. From these results, we concluded that SH2 domain of Sck is an essential region for the binding to KDR.

Unexpectedly, the β-galactosidase activities of the transformants having Sck PTB-CH-SH2 were much

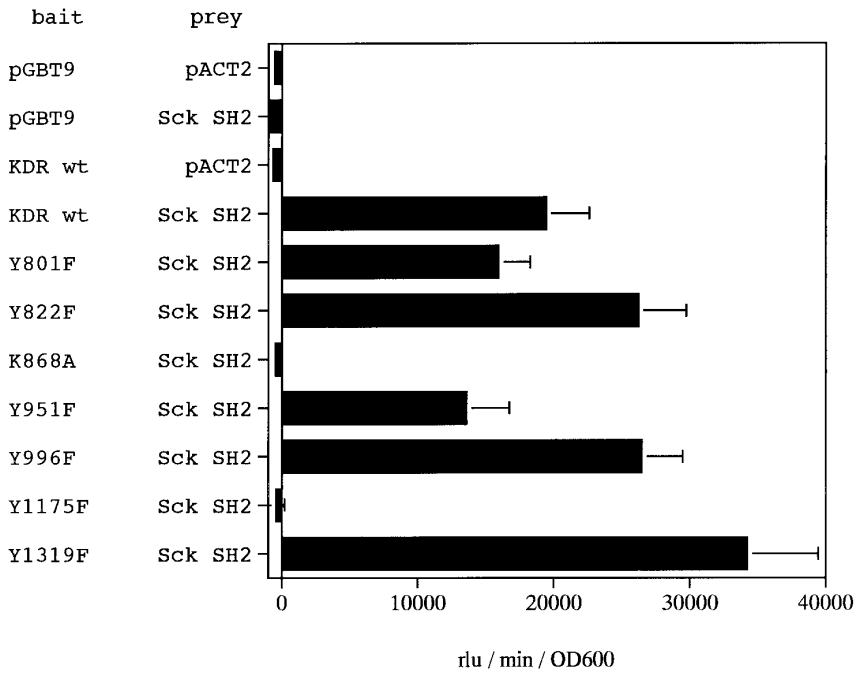

FIG. 5. Sck SH2 binds to Y1175 of KDR. The prey Sck SH2 plasmid was co-transformed with the mutants of KDR into yeast Y187. The β-galactosidase activities of the resulted transformants were assayed using chemiluminescent Galacton Star as a substrate. Values represent the mean ± SD of triplicate determinations expressed in relative light units divided by reaction time (60 min) and optical density.

higher than those of other transformants. This suggests that although PTB domain and CH domain are not necessary for the binding to KDR and Flt-1, they may stabilize the structure of SH2 domain and lead to the firm binding. However, further study must be needed to confirm this possibility.

Sck SH2 domain binds to Y1175 of KDR. To determine the tyrosine residues of KDR and Flt-1 responsible for Sck binding, we co-transformed the SH2 domain of Sck and either KDR or Flt-1 mutant constructs whose tyrosine residues were individually mutated to phenylalanine (Fig. 5). β-galactosidase activity of each transformant was quantified using Galacton-Star as substrate. As shown in Fig. 5, the mutation of kinase domain (K868A of KDR and K861A of Flt-1) causes complete loss of the activity. Substitution of Y1175 of KDR resulted in about 98% lowering of β-galactosidase activity. However, substitution of tyrosine residues of Flt-1 did not cause a significant lowering of β-galactosidase activity. These results indicate that Y1175 of KDR is responsible for the binding to Sck. We further examined the effect of a triple mutant of Flt-1 (Y1309F, Y1327F, and Y1333F), however, it did not lead to a significant lowering of β-galactosidase activity (data not shown). There might be several tyrosine residues of Flt-1 that are responsible for the binding to the SH2 domain of Sck. Further analysis is required to identify the responsible tyrosine residues of Flt-1.

In this paper, we reported Sck as a binding protein to activated KDR and Flt-1. SH2 domain but PTB domain of Sck is responsible for the binding. We showed Y1175 of KDR is responsible for the binding of the SH2 domain of Sck to KDR, but we could not determine the responsible tyrosine residues of Flt-1. The binding of Sck to the activated EGFR has been reported, however, the responsible tyrosine residues of EGFR are not determined (18). So, this is the first time that the surrounding amino acid sequence of the tyrosine residues to which Sck binds is reported. The most important question that we have to answer is how Sck plays in VEGF signal transduction. Upon EGF stimulation, Sck binds to EGFR and is phosphorylated on its tyrosine residues and Grb2 binds to the phosphorylated Sck (18). Therefore, Sck might play some roles in MAPK activation as an adapter protein in EGF system, so that it might also play some roles in VEGF system.

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REFERENCES

1. Thomas, K. A. (1996) *J. Biol. Chem.* **271,** 603–606.
2. Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Siegel, N. R., Leimgruber, R. M., and Feder, J. (1989) *J. Clin. Invest.* **84,** 1470–1478.
3. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. (1994) *J. Biol. Chem.* **269,** 26988–26995.
4. Arkonac, B. M., Foster, L. C., Sibinga, N. E. S., Patterson, C.,

Lai, K., Tsai, J., Lee, M., Perrella, M. A., and Haber, E. (1998) *J. Biol. Chem.* **273,** 4400–4405.

5. Bryant, C. E., Appleton, I., and Mitchell, J. A. (1998) *Life Sci.* **62,** 2195–2201.
6. Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. (1998) *Am. J. Physiol.* **274,** H1054–H1058.
7. Senger, D. R., Claffey, K. P., Benes, J. E., Perruzzi, C. A., Sergiou, A. P., and Detmar, M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 13612–13617.
8. Abedi, H., and Zachary, I. (1997) *J. Biol. Chem.* **272,** 15442–15451.
9. Kevil, C. G., Payne, D. K., Mire, E., and Alexander, J. S. (1998) *J. Biol. Chem.* **273,** 15099–15103.
10. Esser, S., Lampugnani, M. G., Corada, M., Dejana, E., and Risau, W. (1998) *J. Cell Sci.* **111,** 1853–1865.
11. Guo, D., Jia, Q., Song, H. Y., Warren, R. S., and Donner, D. B. (1995) *J. Biol. Chem.* **270,** 6729–6733.
12. Sawano, A., Takahashi, T., Yamaguchi, S., and Shibuya, M. (1997) *Biochem. Biophys. Res. Commun.* **238,** 487–491.
13. Cunningham, S. A., Waxham, M. N., Arrate, P. M., and Brock, T. A. (1995) *J. Biol. Chem.* **270,** 20254–20257.
14. Cunningham, S. A., Arrate, M. P., Brock, T. A., and Waxham, M. N. (1997) *Biochem. Biophys. Res. Commun.* **240,** 635–639.
15. Kroll, J., and Waltenberger, J. (1997) *J. Biol. Chem.* **272,** 32521–32527.
16. Igarashi, K., Isohara, T., Kato, T., Shigeta, K., Yamano, T., and Uno, I. (1998) *Biochem. Biophys. Res. Commun.* **246,** 95–99.
17. Bartel, P. L., Chien, C.-T., Sternglanz, R., and Fields, S. (1993) *Cell. Interact. Dev. Pract. Approach,* 153–179.
18. Nakamura, T., Muraoka, S., Sanokawa, R., and Mori, N. (1998) *J. Biol. Chem.* **273,** 6960–6967.
19. Kroll, J., and Waltenberger, J. (1997) *J. Biol. Chem.* **272,** 32521–32527.