The Carboxyl Terminus Controls Ligand-dependent Activation of VEGFR-2 and Its Signaling

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Vascular endothelial growth factor receptor-2 (VEGFR-2/FLK-1) is a receptor tyrosine kinase whose activation stimulates angiogenesis. We recently generated a chimeric VEGFR-2 in which the extracellular domain of VEGFR-2 was replaced with the extracellular domain of human colony stimulating factor-1 receptor and expressed in endothelial cells. To study the contribution of the carboxyl terminus to activation of VEGFR-2, we created a panel of truncated receptors in which the carboxyl terminus of VEGFR-2 was progressively deleted. Removal of the entire carboxyl terminus eliminated activation of VEGFR-2, its ability to activate signaling proteins, and its ability to stimulate cell proliferation. The carboxyl terminus-deleted VEGFR-2 exhibited impaired ligand-dependent down-regulation and inhibited the activation of wild-type receptor in a dominant-negative fashion. Furthermore, introducing the carboxyl terminus of another receptor, i.e., VEGFR-1, restored the ligand-dependent activation of the carboxyl terminus-deleted VEGFR-2 and its ability to stimulate cell proliferation. Our findings suggest that the carboxyl terminus of VEGFR-2 plays a critical role in VEGFR-2 activation, its ability to activate signaling proteins, and its ability to induce biological responses. The presence of at least 57 amino acids at the carboxyl terminus of VEGFR-2 are required for VEGFR-2 activation. Thus, we propose that the carboxyl terminus is required for activation of VEGFR-2, and absence of the carboxyl terminus renders VEGFR-2 inactive.

Vascular endothelial growth factor receptor-2 (VEGFR-2/FLK-1/KDR) is a receptor tyrosine kinase (RTK) that is expressed predominantly on endothelial cells. Activation of VEGFR-2 is required for normal embryonic vascular development and pathological angiogenesis (1–3). The engagement of VEGFR-2 catalytic activity by ligand stimulation initiates the intracellular signal relay of VEGFR-2 and angiogenesis (4, 5). However, fundamental issues regarding this key event have not been adequately addressed. Recent structure resolution of RTKs, including the insulin receptor (6) and fibroblast growth factor receptor (7), demonstrate that these receptors bind ATP-Mg2+ and phosphorylate at least one of three tyrosine sites in the activation loop. These crystal structures reveal that in the unstimulated state, the activation loop orients these tyrosine sites toward the activation sites of the enzyme and thereby sterically prevents them from binding to ATP-Mg2+. It is suggested that phosphorylation of these tyrosines in the catalytic loop orients the inhibitory loop away from the active site, enabling the RTK to bind ATP-Mg2+ and efficiently phosphorylate substrates. As a result of ligand binding, the receptor reorients such that it is now able to transphosphorylate and thereby activates each other’s kinase activity (8, 9). Thus, structural data combined with many elegant biochemical studies of RTKs provide direct evidence for the regulation of kinase activation by the activation loop.

To date, the contribution of carboxyl and juxtamembrane (JM) domains to RTK activation is less understood. The recent resolution of the crystal structure of Tie-2 reveals that the activation loop, unlike other RTKs, is in an inhibitory conformation, and its carboxyl terminus may block access to the substrate binding site (10). This finding suggests that other domains of RTKs, such as JM and carboxyl domains, may regulate the catalytic activation of RTKs. Of the published crystal structure of RTKs resolved to date, the JM and carboxyl domains are not included (6, 7, 10, 11). Thus, the contribution of these domains to activation of RTKs remains unknown.

The recent resolution of the crystal structure of VEGFR-2 revealed that the activation loop of VEGFR-2 is highly disordered and resides in an inhibitory conformation (12). This crystal structure, however, was resolved without its carboxyl terminus. We postulated that the presence of the carboxyl terminus of VEGFR-2 is required for its activation, and the absence of it prevents VEGFR-2 from changing its conformation from an inhibitory to an active conformation. In the present study, we have tested the involvement of the carboxyl terminus of VEGFR-2 in its ligand-dependent activation. Our results demonstrate that partial deletion of the carboxyl domain of VEGFR-2 preserves its ligand-dependent activation. However, deletion of the entire carboxyl terminus abolished its ligand-dependent autophosphorylation, its ability to activate signaling proteins, and its ability to stimulate biological responses. Introducing the carboxyl domain of VEGFR-1/FLT-1 restored the ligand-dependent activation of the carboxyl terminus-deleted receptor and its ability to stimulate cell proliferation. Collectively, these results suggest that the presence of carboxyl terminus of VEGFR-2 is necessary for maximal autophosphorylation of VEGFR-2 and its ability to induce biological responses.

MATERIALS AND METHODS

Reagents and Antibodies—Human recombinant colony stimulating factor (CSF)-1 was purchased from R&D. Mouse anti-phosphotyrosine...
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(PY-20), anti-phospholipase C (PLC)-γ, and anti-mouse and anti-rabbit secondary antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-MAPK and anti-phospho-MAPK were purchased from New England BioLabs (Boston, MA). Rabbit anti-phospho-PLC-γ and phosphophoryrosine-1173 VEGF-2, phosphotyrosine 1052 and 1057 antibodies were purchased from BIOSOURCE (Camarillo, CA). Rabbit anti-VEGF-2 antibody was made to amino acids corresponding to the kinase insert or carboxyl terminus of VEGF-2 (13). Mouse anti-phosphoryrosine (4G10) and rabbit phospho-tyrosine-1212 VEGF-2 antibodies were purchased from UBI (Lake Placid, NY).

Cell Lines—Porcine aortic endothelial (PAE) cells expressing CSF-1R/FLK-1 Receptor (CKR) and truncated receptors were established by a retroviral system as described previously (13). Briefly, cDNA encoding for CKR was cloned into retroviral vector pLNCX2, and transfected into 293-GPG cells. Viral supernatants were collected for 7 days, concentrated by centrifugation, and used as described previously (14). Site-directed Mutagenesis—The VEGF-2 chimera (CKR) was used as a template to construct the carboxyl terminus truncated CKR. The truncations were made using PCR-based site-directed mutagenesis (14, 15). The antisense PCR primer for ΔCKR/152 was GTGCATGATGTTGGCATATTCAG and the sense primer for ΔCKR/152 was GTGCATGATGTTGGCATATTCAGATGGATTT. The antisense primer for ΔCKR/152 was GTGCATGATGTTGGCATATTCAGATGGATTT. ΔCKR/212 lacks the entire carboxyl terminus; 630 bp were deleted (3470–4100 bp). Its carboxyl terminus contains two amino acids (Glu-1156 and His-1157) following its kinase domain. ΔCKR/152 was generated by deleting 460 bp of carboxyl terminus of VEGF-2 corresponding to 3640–4100 bp. The carboxyl terminus of ΔCKR/152 contains 60 amino acids including tyrosines 1173 and 1212. ΔCKR/157 was made by deleting 473 bp (3827–4100). The carboxyl terminus of ΔCKR/157 contains 55 amino acids including tyrosine 1173. The resultant truncated receptors were verified by sequencing and were subsequently cloned into pLNCX2 or pLXSN vector by NotI and SalI sites. To generate the carboxyl terminus of VEGF-2 (C-tail) the entire carboxyl terminus (630 bp from 3470 to 4100) of VEGF-2 PCR was amplified. The sense primer was also designed to contain a Kozak and 16 amino acids downstream of the carboxyl terminus. ΔCKR/152 generated a receptor that moves the entire carboxyl terminus of VEGFR-2, including tyrosines 1173 and 1212. Deletion of 152 amino acids generates a truncated receptor with two tyrosine sites at the carboxyl terminus, namely tyrosines 1173 and 1212. Deletion of 157 amino acids generates a truncated receptor with two tyrosine sites at the carboxyl terminus, namely tyrosines 1173 and 1212, but it eliminates tyrosines 1221, 1303, 1307, and 1317. Deletion of 157 amino acids generates a truncated receptor with only one tyrosine site, namely 1173 at its carboxyl terminus. Finally, deletion of 212 amino acids removes the entire carboxyl terminus of VEGFR-2, including tyrosines 1173 and 1212. The truncated receptors are herein called ΔCKR/152, ΔCKR/157, and ΔCKR/212. The truncated receptors were expressed in PAE cells using a retroviral system. PAE cells individually expressing wild-type receptor and truncated receptors were lysed and equal amounts of proteins were subjected to Western blot analysis using anti-phosphotyrosine antibodies. Equal amounts of protein from each lysate were immunoprecipitated using a rabbit polyclonal anti-VEGF-2 antibody (1410). Antigen-antibody complexes were allowed to bind protein A-Sepharose conjugated beads for 1 h at 4 °C, followed by three washes with 1.0 ml of EB buffer. Immunocomplexes were subjected to Western blot analysis using streptavidin-horseradish peroxidase conjugate and were detected by ECL system (Amersham Biosciences).

RESULTS

Carboxylic Terminus of VEGFR-2 Is Important for Its Ligand-Dependent Activation—To study the selective ligand-dependent biological and biochemical functions of VEGF-2 in endothelial cells, we recently generated a chimeric VEGF-2 (herein called CKR) in which the extracellular domain of human CSF-1R was fused to the transmembrane and cytoplasmic domains of mouse VEGFR-2 and was stably expressed in PAE cells (4). The carboxyl tail of VEGF-2 contains 214 amino acids with six potential tyrosine phosphorylation residues (Fig. 1A). In this study, we have used CKR to generate a panel of truncated receptors in which the carboxyl terminus of CKR was progressively deleted by 152, 157, and 212 amino acids. As presented in Fig. 1A, deletion of 152 amino acids generates a receptor with two tyrosine sites at the carboxyl terminus, namely tyrosines 1173 and 1212, but it eliminates tyrosines 1221, 1303, 1307, and 1317. Deletion of 157 amino acids generates a truncated receptor with only one tyrosine site, namely 1173 at its carboxyl terminus. Finally, deletion of 212 amino acids removes the entire carboxyl terminus of VEGFR-2, including tyrosines 1173 and 1212. The truncated receptors are herein called ΔCKR/152, ΔCKR/157, and ΔCKR/212. The truncated receptors were expressed in PAE cells using a retroviral system. PAE cells individually expressing wild-type receptor and truncated receptors were lysed and equal amounts of proteins were subjected to Western blot analysis using an anti-VEGF-2 antibody which specifically recognizes the kinase...
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The phosphorylation of at least two highly conserved and specific tyrosine residues at the activation loop of RTKs is suggested to control ligand-dependent activation of RTKs (16, 17). Phosphorylation of tyrosines 1052 and 1057, located in the activation loop of VEGFR-2, is required for its activation and signaling (18). To directly test the contribution of carboxyl tail toward ligand-dependent activation of CKR, we have used a recently developed anti-phospho-specific antibody directed against phospho-tyrosines 1052 and 1057 residues of VEGFR-2. The initial analysis and characterization of this antibody showed that this antibody selectively recognizes phospho-tyrosines 1052 and 1057, because the mutation of these tyrosine sites abolished the ability of this antibody to detect tyrosine phosphorylated CKR (data not shown). To test whether tyrosines 1052 and 1057 of CKR are phosphorylated in vivo, we used recently developed anti-phospho-specific tyrosine antibodies. To this end, cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, or ΔCKR/212 were stimulated with CSF-1 for 10 min, and total cell lysates were subjected to Western blot analysis using an anti-phospho-CKR antibody for protein level (D).

**FIG. 2. Lack of carboxyl terminus impairs the ligand-dependent activation of VEGFR-2.** Serum-starved PAE cells individually expressing either wild-type CKR, ΔCKR/152, ΔCKR/157, or ΔCKR/212 were treated with CSF-1, and total cell lysates were immunoprecipitated with an anti-VEGFR-2 antibody that specifically recognizes the kinase insert of VEGFR-2. The immunoprecipitated proteins were subjected to Western blot analysis using an anti-phospho-tyrosine antibody (A). B, the same membrane was reprobed with an anti-VEGFR-2 antibody for protein level. C, serum-starved PAE cells expressing either wild-type CKR, ΔCKR/152, ΔCKR/157, or ΔCKR/212 were treated with CSF-1 for 10 min, and total cell lysates were subjected to Western blot analysis using an anti-phospho-tyrosine 1052/1057 VEGFR-2 antibody. The same membrane was reprobed with an anti-VEGFR-2 antibody for protein level (D).

![Diagram](image.png)

**FIG. 1. Schematic presentation and cell surface expression of ΔCKRs.** The carboxyl terminus-deleted chimeric VEGFR-2s (ΔCKRs) were constructed using PCR-based, site-directed mutagenesis. Deleting 152 and 157 amino acids from the carboxyl tail of VEGFR-2 created ΔCKR/152 and ΔCKR/157, respectively. ΔCKR/212 lacks the entire carboxyl terminus with only two amino acids remaining at its carboxyl tail (A). Equal numbers of PAE cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, and ΔCKR/212 were lysed, and total cell lysates were subjected to Western blot analysis using an anti-VEGFR-2 antibody that specifically recognizes the kinase insert of VEGFR-2 (B). PAE cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, and ΔCKR/212 were cell surface labeled with a membrane-impermeable biotin analogue. Cells were lysed and immunoprecipitated with an anti-VEGFR-2 antibody. The immunoprecipitated proteins were probed with anti-streptavidin-peroxidase (anti-SA-HRP) antibody (C).

insert domain of VEGFR-2. Fig. 1B shows that the wild-type receptor and the truncated receptors are expressed at relatively similar levels in PAE cells. In addition, the expression of these truncated receptors at the surface of PAE cells was also analyzed by labeling cells with biotin. As presented in Fig. 1C, all the truncated receptors were expressed at the surface of PAE cells.

To test the relative significance of carboxyl terminus to ligand-dependent autophosphorylation of CKR, we first analyzed the ability of truncated CKRs to undergo ligand-dependent autophosphorylation in vivo. Fig. 2A shows that the deletion of 152 and 157 amino acids at the carboxyl terminus of CKR had no significant effect on the ligand-dependent autophosphorylation of VEGFR-2, as detected by anti-phosphotyrosine antibody. However, deletion of 212 amino acids at the carboxyl terminus of the receptor, which entirely removes its carboxyl tail, abolished its ligand-dependent autophosphorylation (Fig. 2A).

The phosphorylation of at least two highly conserved and specific tyrosine residues at the activation loop of RTKs is suggested to control ligand-dependent activation of RTKs (16, 17). Phosphorylation of tyrosines 1052 and 1057, located in the activation loop of VEGFR-2, is required for its activation and signaling (18). To directly test the contribution of carboxyl tail toward ligand-dependent activation of CKR, we have used a recently developed anti-phospho-specific antibody directed against phospho-tyrosines 1052 and 1057 residues of VEGFR-2. The initial analysis and characterization of this antibody showed that this antibody selectively recognizes phospho-tyrosines 1052 and 1057, because the mutation of these tyrosine sites abolished the ability of this antibody to detect tyrosine phosphorylated CKR (data not shown). To test whether tyrosines 1052 and 1057 of CKR are phosphorylated in vivo, we used recently developed anti-phospho-specific tyrosine antibodies. To this end, cells expressing wild-type CKR, F1212/CKR, and E1212/CKR were stimulated with CSF-1 for 10 min, and total cell lysates were subjected to Western blot analysis using phospho-specific tyrosine 1212 antibody. As demonstrated in Fig. 3A,
the anti-phospho-specific tyrosine 1212 antibody recognizes only wild-type CKR stimulated with CSF-1 but not the F1212/CKR and E1212/CKR. This suggests that tyrosine 1212 of murine VEGFR-2 is phosphorylated in response to ligand stimulation in endothelial cells. To test phosphorylation of tyrosine 1173, we used cells expressing wild-type CKR, F1173/CKR, ΔCKR/152, and ΔCKR/157. As shown in Fig. 3C, a phosphospecific tyrosine-1173 antibody selectively detected the ligand-stimulated CKR, ΔCKR/152, and ΔCKR/157 but not F1173/CKR. Thus, both tyrosines 1212 and 1173 of VEGFR-2 are phosphorylated in endothelial cells and may participate in ligand-dependent activation of VEGFR-2 and/or in its downstream signaling.

We have recently shown that the presence of tyrosine 1212 in the context of full-length chimeric VEGFR-2 is required for maximal ligand-dependent autophosphorylation of VEGFR-2 (14). Phosphorylation of tyrosine 1173 on mouse VEGFR-2 along with tyrosine 799 is involved in the recruitment and activation of phosphatidylinositol 3-kinase (PI3K) (19). To test the potential involvement of tyrosines 1173 and 1212 to the ligand-dependent autophosphorylation of VEGFR-2 in the context of ΔCKR/152, which contains only two tyrosine phosphorylation sites, namely tyrosine 1173 and 1212, we mutated these tyrosines to phenylalanine (Fig. 3E). The tyrosine mutant ΔCKR/152 is herein called ΔCKR/152/F2. Analysis of the ligand-dependent autophosphorylation of ΔCKR/152/F2 revealed that this receptor was unexpectedly able to undergo ligand-dependent autophosphorylation with no apparent decrease in its autophosphorylation (Fig. 3F). Altogether, these data suggest that although phosphorylation of tyrosine 1212 in the context of full-length VEGFR-2 directly or indirectly participates in the ligand-dependent autophosphorylation of VEGFR-2, phosphorylation of tyrosine 1212 in the context of carboxyl terminus-deleted VEGFR-2 appears to play a compensatory role in its ligand-dependent autophosphorylation. These data suggest that 157 amino acids at the carboxyl terminus are dispensable for full activation of VEGFR-2. At least 57 amino acids at carboxyl tail are required for ligand-dependent tyrosine phosphorylation of VEGFR-2.

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The carboxyl terminus is a requirement for ligand-dependent down-regulation of VEGFR-2. PAE cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, and ΔCKR/212 (A–D) were starved in serum-free and methionine/cystine-free medium and then were labeled with [35S]methionine/cystine (75 μCi/ml) for 3 h. Cells were stimulated with CSF-1 for the indicated periods of time (−30 min to 30 min) before lysis and protein detection using the Image Station (Kodak). E, the graph represents the average of two separate experiments.

**Fig. 3. Tyrosines 1173 and 1212 of VEGFR-2 are phosphorylated in vivo.** Equal numbers of serum-starved, semiconfluent PAE cells expressing CKR, F1212/CKR, and E1212/CKR were either not stimulated or stimulated with 40 ng/ml CSF-1 for 10 min, washed, and lysed. Total cell lysates were subjected to Western blot analysis using an anti-phospho-tyrosine 1212 antibody (A), B, the same membrane was reprobed with anti-VEGFR-2 antibody. Serum-starved cells expressing CKR, F1212/CKR, and E1212/CKR were either not stimulated or stimulated with 40 ng/ml CSF-1 for 10 min, washed, and lysed. Total cell lysates were subjected to Western blot analysis using an anti-phosphotyrosine antibody (B). C, total cell lysates were subjected to Western blot analysis using an anti-phosphotyrosine 1173 antibody. D, the same membrane was reprobed with an anti-VEGFR-2 antibody. E, schematic representation of ΔCKR/152 and ΔCKR/152/F1173/F2121. The ΔCKR/152/F1173/F2121 herein is named ΔCKR/152/F2. Serum-starved cells expressing CKR, ΔCKR/152, and ΔCKR/152/F2 were stimulated with CSF-1 for 10 min as in C. F, total cell lysates were subjected to Western blot analysis using an anti-phosphotyrosine antibody. G, the same membrane was reprobed with anti-VEGFR-2 antibody.

**Fig. 4. The carboxyl terminus is a requirement for ligand-dependent down-regulation of VEGFR-2.** PAE cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, and ΔCKR/212 (A–D) were starved in serum-free and methionine/cystine-free medium and then were labeled with [35S]methionine/cystine (75 μCi/ml) for 3 h. Cells were stimulated with CSF-1 for the indicated periods of time (−30 min to 30 min) before lysis and protein detection using the Image Station (Kodak). E, the graph represents the average of two separate experiments.
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**Fig. 5. Effect of deletion of carboxyl terminus of VEGFR-2 on activation of PLC-1, AKT, and MAPK.** Equal numbers of serum-starved, semiconfluent PAE cells expressing either wild-type CKR, ΔCKR/152, ΔCKR/157, or ΔCKR/212 were treated with CSF-1 (40 ng/ml) for 10 or 30 min, washed, and lysed. Total cell lysates were subjected to Western blot analysis using an anti-phospho-PLC-1 antibody (A), phospho-AKT antibody (B), and anti-MAPK antibody (E). The same membranes were reprobed with anti-PLC-1 antibody (D), anti-AKT antibody (C), and anti-MAPK antibody (E) for protein levels. Cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, or ΔCKR/212 were unstimulated (−) or stimulated with CSF-1 (+) (40 ng/ml) for 10 min; cells were washed and lysed. Total cell lysates were either immunoprecipitated with GST-N-SH2-PLC-1 (G) or with anti-phospho-PI3K antibody (H), and membranes were immunoblotted with anti-VEGFR-2 antibody.

2 h of ligand stimulation (Fig. 4, D and E). Collectively, these results suggest that the carboxyl tail of VEGFR-2 is required for full ligand-dependent down-regulation of VEGFR-2. Because removal of tyrosines 1212 and 1173 greatly impairs the down-regulation of VEGFR-2, it is highly possible that phosphorylation of one or both of these tyrosines may recruit signaling proteins that might contribute to down-regulation of VEGFR-2.

**Requirement of Carboxyl Terminus for VEGFR-2-mediated Activation of Signaling Proteins—**To test the ability of the carboxyl terminus truncated receptors to activate signaling proteins, we have analyzed the activation of PLC-1, Akt, and MAPK. As presented in Fig. 5A, stimulation of ΔCKR/152 and ΔCKR/157 resulted in the activation of PLC-1 comparable with that of wild-type receptor. This suggests that tyrosine residues located within this region, including 1221, 1303, and 1308, are not required for VEGFR-2-mediated PLC-1 activation. Unlike ΔCKR/152 and ΔCKR/157, the ability of ΔCKR/212 to stimulate PLC-1 significantly was compromised, and phosphorylation of PLC-1 by this receptor was not observed (Fig. 5A). Similarly, both ΔCKR/152 and ΔCKR/157 but not ΔCKR/212 were able to stimulate AKT phosphorylation (Fig. 5C). Although ΔCKR/212 failed to undergo ligand-dependent autophosphorylation or to stimulate PLC-1 and AKT, it was still able to stimulate MAPK phosphorylation. It should be noted that the ability of ΔCKR/212 to stimulate MAPK phosphorylation was slightly reduced compared with that of wild-type CKR, ΔCKR/152, and ΔCKR/157 (Fig. 5E).

To further analyze the ability of carboxyl tail-truncated CKRs to activate signaling proteins, we measured their ability to associate with PLC-1 and phospho-AKT antibody (ΔCKR/152, ΔCKR/157, and ΔCKR/212 were treated with different concentrations of CSF-1, and DNA synthesis was measured by [H]thymidine uptake. The results are expressed as the mean of (cpm/well) ± S.D. of quadruplicates. The data are expressed as a ratio of stimulated over nonstimulated samples.

**Fig. 6. Effect of deletion of carboxyl terminus in VEGFR-2-mediated cell proliferation.** Serum-starved PAE cells expressing wild-type CKR, ΔCKR/152, ΔCKR/157, and ΔCKR/212 were treated with different concentrations of CSF-1 and ΔCKR/212 are efficiently associated with the GST-N-SH2-PLC-1 (Fig. 5G). We finally assessed the ability of wild-type CKR and the carboxyl terminus-deleted CKRs to bind with phospho-PI3K. The result showed that ΔCKR/152 and ΔCKR/157, but not ΔCKR/212, are able to associate with the phospho-PI3K (Fig. 5H).

In summary, our results show that the carboxyl terminus of VEGFR-2 influences both its ligand-dependent activation and its ability to associate with signaling proteins.

**Role of Carboxyl Terminus in VEGFR-2-mediated Endothelial Cell Proliferation—**To determine the ability of ΔCKR/152, ΔCKR/157, and ΔCKR/212 to stimulate biological responses in endothelial cells, we subjected PAE cells expressing the truncated receptors to proliferation and differentiation assays. As presented in Fig. 6, stimulation of ΔCKR/152 and ΔCKR/157 induced cell proliferation in a CSF-1-dependent manner. However, the ability of ΔCKR/157 to stimulate cell proliferation was partially reduced. In contrast, stimulation of ΔCKR/212 resulted in no significant cell proliferation. Similarly, ΔCKR/152 and ΔCKR/157 but not ΔCKR/212 were able to induce morphological changes in PAE cells (data not shown). Altogether, these results suggest that the 57 amino acids in the carboxyl terminus of VEGFR-2 are critical for its ability to stimulate endothelial cell proliferation and differentiation. Furthermore, these results demonstrate that MAPK activation is not essential for VEGFR-2-induced cell proliferation or differentiation, because ΔCKR/212 is able to stimulate MAPK activation but fails to stimulate cell proliferation and differentiation.

**ΔCKR/212 Acts as a Dominant-Negative Receptor—**Because ligand-dependent autophosphorylation of ΔCKR/212 is severely impaired, it is highly suggestive that this receptor may act in a dominant-negative fashion when co-expressed with wild-type CKR. To this end, we tested the ability of ΔCKR/212 to attenuate the ligand-dependent phosphorylation of CKR by co-expressing ΔCKR/212 with CKR. As presented in Fig. 7A, co-expression of ΔCKR/212 with CKR significantly decreased...
the ligand-dependent tyrosine phosphorylation of CKR. This suggests that deletion of the carboxyl tail of VEGFR-2 may serve as a means to attenuate VEGFR-2 function in pathological conditions where VEGFR-2 activity is elevated.

To further understand the molecular mechanism by which carboxyl tail contributes to VEGFR-2 activation, we have coexpressed the carboxyl tail of VEGFR-2 with CKR in PAE cells (Fig. 7C). Our data show that coexpression of the carboxyl tail of VEGFR-2 with the wild-type receptor does not impair the ligand-dependent tyrosine phosphorylation of CKR (Fig. 7D). This suggests that the potential interaction between the carboxyl terminus and other domains of VEGFR-2 requires the intact carboxyl terminus.

The Carboxyl Tail of VEGFR-1 Rescues the Lack of Ligand-dependent Autophosphorylation of ΔCRK212 and Its Ability to Stimulate Proliferation of Endothelial Cells—Our results demonstrate that complete deletion of the carboxyl tail ablates the autophosphorylation of VEGFR-2 and its ability to stimulate tyrosine phosphorylation of signaling proteins and induce cell proliferation. To determine whether the carboxyl terminus of another VEGFR-2 family members such as VEGFR-1 (FLT-1) could be substituted for sequence in the carboxyl terminus of VEGFR-2, we constructed a CKR-VEGFR-1 chimera in which the carboxyl terminus of VEGFR-2 was replaced with that of VEGFR-1 (Fig. 8A). The chimeric receptor is called CKR/cFLT-1 and was expressed in PAE cells (Fig. 8B). We used this carboxyl tail-swapped receptor and asked whether the carboxyl tail-swapped receptor also is able to activate MAPK but not PLC-1 (Fig. 8C). Thus, the carboxyl tail-swapped receptor also was able to activate MAPK but not PLC-1 (Fig. 8E and G). Thus, the carboxyl tail of VEGFR-1, although able to rescue the lack of tyrosine phosphorylation of ΔCRK212, failed to stimulate activation of PLC-1.

Deletion of the entire carboxyl tail of CKR abrogated its ability to stimulate endothelial cell proliferation (Fig. 6). Thus, we addressed whether the carboxyl tail-swapped receptor (CKR-c-FLT-1) could rescue the inability of ΔCRK212 to stimulate cell proliferation. To this end, we subjected PAE cells...
expressing either ΔCKR/212 or CKR-c-FLT-1 to a proliferation assay. The result demonstrates that the carboxyl tail-swapped receptor is able to stimulate proliferation of PAE cells (Fig. 9). This is consistent with the idea that the presence of carboxyl tail of VEGFR-2 is required for its full activation and its ability to stimulate biological functions.

**DISCUSSION**

The engagement of VEGFR-2 catalytic activity by ligand stimulation initiates the intracellular signal relay and subsequent cellular responses (4, 5, 18). Data presented from this report demonstrate that deletion of 212 amino acids in the carboxyl terminus of VEGFR-2 severely impairs the ability of VEGFR-2 to undergo ligand-dependent autophosphorylation and to stimulate signaling proteins. Our study provides the most compelling data indicating a critical role for the carboxyl terminus of VEGFR-2 in its ligand-dependent activation. Only 57 amino acids from the carboxyl terminus of VEGFR-2 are sufficient to preserve the ligand-dependent activation of VEGFR-2 and its ability to stimulate biological responses.

The recent crystal structure of VEGFR-2, although resolved without its carboxyl tail, revealed that the activation loop of VEGFR-2 is highly disordered and it is in an inhibitory “gate-close” conformation (12). The conformation of the activation loop of RTKs is suggested to regulate the kinase activation of RTKs by adapting either a “gate-open” or “gate-close” conformation, allowing ATP and peptide substrates to bind or not bind to it, respectively (22–24). This observation was rather surprising because the inhibitory conformation was only observed for RTKs in the unphosphorylated and inactive state. Interestingly, the deletion of kinase insert domain of VEGFR-2 did not change its inhibitory conformation (12). One possible explanation for the unexpected conformation of the activation loop of VEGFR-2 is that other domains of VEGFR-2, such as carboxyl terminus, may regulate its conformation. Our observations demonstrate that the presence of at least 57 amino acids at the carboxyl terminus of VEGFR-2 is required for full activation of VEGFR-2. However, the deletion of these 57 amino acids abolished the ligand-dependent activation of VEGFR-2. Introducing the carboxyl terminus of another RTK, namely FLT-1/VEGFR-1, rescued the ligand-dependent autophosphorylation of VEGFR-2 and cell proliferation. Altogether, these observations strongly implicate the carboxyl terminus in VEGFR-2 activation.

**Fig. 9. Activation of ΔCKR/cFLT-1 induces cell proliferation.** Serum-starved PAE cells expressing ΔCKR/212 or CKR-cFLT-1 were treated with different concentrations of CSF-1, and DNA synthesis was measured by [H]thymidine uptake. The results are expressed as the mean of (cpm/well) ± S.D. of quadruplicates. The data are expressed as a ratio of stimulated over nonstimulated samples.

The idea that conformational changes of the activation loop of RTKs from a “gate-close” to “gate-open” state determines the autophosphorylation, and activation of RTKs is strongly supported by information obtained from the crystal structures of several RTKs including the fibroblast growth factor receptor, insulin receptor, and Src family kinases (6, 7, 25). However, new reports suggest that an increasingly intricate and complex mode of regulation may govern the kinase activation of RTKs. For instance, the recent resolution of the crystal structure of Tie-2 revealed that the activation loop is in an inhibitory conformation, and its carboxyl terminus blocks the activation loop by blocking ATP and substrate binding (10). The deletion of the carboxyl terminus of other RTKs including epidermal growth factor receptor (26, 27), CSF-1 receptor (28), hepatocyte growth factor receptor/Met (29), and platelet-derived growth factor receptor (30) has been shown to affect their ligand-dependent activation. In some other cases, deletion of the carboxyl terminus of RTKs has been reported to enhance the autophosphorylation and biological responses (31, 32). Collectively, these observations strongly argue that carboxyl tails of RTKs are involved in regulation of kinase activation of RTKs. In addition to carboxyl terminus, the JM domains of RTKs have also been linked to kinase activation of RTKs. For instance, the presence JM domains of EphB2, c-kit, and Musk are required for their catalytic activation (33–35).

How does the carboxyl terminus of VEGFR-2 modulate its activation? One possibility is that the carboxyl terminus in the unstimulated state directly interacts with the activation loop. This interaction prevents substrate binding to the activation loop and phosphorylation of the conserved tyrosine sites located in the activation loop. The contribution of the carboxyl tail to kinase activation of RTKs by this mechanism has been suggested recently for Tie-2 (10, 36) and hepatocyte growth factor receptor (29). The carboxyl terminus of VEGFR-2, in
particular tyrosine phosphorylation sites within this domain, may directly or indirectly modulate VEGFR-2 activation by recruiting proteins such as tyrosine phosphatases that may promote dephosphorylation of VEGFR-2.

Thus, it is possible that the carboxyl terminus influences the overall tertiary structure of VEGFR-2, and lack of carboxyl terminus may interfere with adaptation of VEGFR-2 from an inhibitory “gate-close” conformation to an active “gate-open” conformation. The data presented in this study support this possibility. First, deletion of the entire carboxyl tail abolishes autophosphorylation of VEGFR-2. Second, introducing the carboxyl terminus of VEGFR-1 with the carboxyl tail-deleted CKR (ΔCKR/212) rescued the autophosphorylation of the receptor. Thus, we propose that in the absence of ligand, VEGFR-2 monomers are inhibited by steric or intramolecular interactions between its activation loop and carboxyl terminus, shown in Fig. 10. In the presence of ligand, VEGFR-2 undergoes dimerization, resulting in conformational changes that may promote release of the carboxyl terminus from the activation loop. This follows the rearrangement of activation loop and autophosphorylation of VEGFR-2. When the carboxyl terminus is not present, the ligand-induced conformational change on the activation loop of VEGFR-2 is not sufficiently accommodated, and thus VEGFR-2 is not autophosphorylated.

In summary, the data presented here demonstrate a critical role for carboxyl terminus in kinase activation and biological functions of VEGFR-2. The carboxyl terminus of VEGFR-2 appears to play two distinct roles: to provide binding sites for signaling proteins, and to contribute to kinase activation of VEGFR-2. Further studies, in particular resolving the crystal structure of VEGFR-2 with presence of its carboxyl terminus, will determine the mechanisms of involvement of the carboxyl terminus in kinase activation of VEGFR-2.

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