The Carboxyl Terminus of VEGFR-2 Is Required for PKC-mediated Down-Regulation

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Vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1) is a receptor tyrosine kinase (RTK) whose activation regulates angiogenesis. The regulatory mechanisms that attenuate VEGFR-2 signal relay are largely unknown. Our study shows that VEGFR-2 promotes phosphorylation of c-Cbl, but activation, ubiquitylation, and down-regulation of VEGFR-2 are not influenced by c-Cbl activity. A structure-function analysis of VEGFR-2 and pharmacological approach revealed that down-regulation of VEGFR-2 is mediated by a distinct mechanism involving PKC. A tyrosine mutant VEGFR-2, defective in PLC- γ 1 activation underwent down-regulation efficiently in response to ligand stimulation, suggesting that activation of classical PKCs are not involved in VEGFR-2 down-regulation. Further studies showed that the ectodomain of VEGFR-2 is dispensable for PKC-dependent down-regulation. Progressive deletion of the carboxyl-terminal domain showed that at least 39 amino acids within the carboxyl-terminal domain, immediately C-terminal to the kinase domain, is required for efficient PKC-mediated down-regulation of VEGFR-2. Mutation of serine sites at 1188 and 1191, within this 39 amino acid region, compromised the ability of VEGFR-2 to undergo efficient ligand-dependent down-regulation. Altogether the results show that the regulatory mechanisms involved in the attenuation of VEGFR-2 activation is mediated by nonclassical PKCs and the presence of serine sites in the carboxyl terminal of VEGFR-2.

INTRODUCTION

Vascular endothelial growth factor receptor-2 (VEGFR-2) is an endothelial cell receptor tyrosine kinase (RTK) whose activation promotes mitogenesis and differentiation of vascular endothelial cells, two cellular events that play important roles in normal and pathological angiogenesis (Risau, 1997; Jain, 2003). In the absence of ligand, most RTKs are catalytically inactive due to the influence of multiple layers of *cis*-acting autoinhibitory mechanisms (Blume-Jensen and Hunter, 2001). Binding of ligand promotes receptor autophosphorylation and initiation of an array of intracellular signaling pathways that regulate a wide range of biological outcomes, including cellular proliferation, differentiation, and survival (Pawson and Nash, 2000).

Accelerated internalization and reduced recycling are the main ligand-induced changes in RTK trafficking that cause a rapid depletion of the cellular receptor pool, a phenomenon termed down-regulation (Sorkin *et al.*, 1992; Marmor and Yarden, 2004). Indeed, multiple regulatory mechanisms have evolved in order to ensure that RTK-induced biological responses occur with the correct magnitude and kinetics. Although their mechanisms of action differ tremendously, the Cbl family of RING finger E3 ubiquitin-protein ligases and the PKC family of serine/threonine kinases has been implicated in the down-regulation of RTK functions. c-Cbl, upon recruitment to specific sites on autophosphorylated RTKs, undergoes phosphorylation-dependent E3-ligase activation and targets them

for proteasomal/lysosomal degradation through ubiquitylation (Levkowitz *et al.*, 1999; Thien and Langdon, 2001). Indeed, several RTKs including epidermal growth factor (EGF) receptor (EGFR) (Levkowitz *et al.*, 1999), platelet-derived growth factor (PDGF) receptor (PDGFR α/β ; Miyake *et al.*, 1998, 1999), colony-stimulating factor-1 receptor (CSF-1R; Lee *et al.*, 1999; Wilhelmsen *et al.*, 2002), c-Met (HGFR; Peschard *et al.*, 2001), and c-Ron (Penengo *et al.*, 2003) are ubiquitylated and undergo degradation in a c-Cbl-dependent manner. These findings highlight the role of Cbl as a rate-limiting factor in ligand-induced down-regulation of these receptors. Thus, Cbl-mediated ubiquitylation provides a mechanism to eliminate activated pools of RTKs via protein degradation, complementing dephosphorylation, and other negative regulatory processes.

In addition to c-Cbl, activation of PKC has emerged as a means by which the expression and activity of RTKs at the cell surface are subject to negative modulation in response to growth factors (Seedorf *et al.*, 1995). Activation of PKC has been shown to negatively regulate the ErbB-1 receptor through direct phosphorylation of the receptor, an event leading to the attenuation of high-affinity ligand binding and intrinsic tyrosine kinase activity (Cochet *et al.*, 1984; Downward *et al.*, 1985; Lund *et al.*, 1990). PKC-induced ectodomain shedding seems to be another prominent mechanism for signal attenuation that is widespread throughout the RTK superfamily. Indeed, the ErbB-4 (Vecchi and Carpenter, 1997; Ni *et al.*, 2003), CSF-1R (Downing *et al.*, 1989), c-Kit (Yee *et al.*, 1993; Cruz *et al.*, 2004), c-Met (Jeffers *et al.*, 1997), TrkA (nerve growth factor receptor; Cabrera *et al.*, 1996), Tie-1 (Yabkowitz *et al.*, 1997), and Axl (O'Bryan *et al.*, 1995) receptor systems are all subject to this mode of negative regulation. In particular, PKC-mediated induction of a novel mechanism known as regulated intramembrane pro-

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teolysis (RIP) has received much attention in recent years. RIP removes receptors from the cell surface through the sequential action of two distinct membrane-localized proteases in which PKC-induced ectodomain shedding precedes the release of a membrane-associated receptor remnant into the cytosol through γ -secretase-dependent cleavage of intramembrane sequences (Ebinu and Yankner, 2002).

In this study, we provide evidence suggesting that c-Cbl E3 ligase activity does not negatively regulate VEGFR-2 signaling through an enhancement in receptor ubiquitylation and turnover. Nonclassical PKC isozymes are components of the signaling mechanisms activated downstream of VEGFR-2 that negatively regulate its signal transduction relay at the receptor level. PKC-induced down-regulation of VEGFR-2 does not involve ectodomain shedding. Instead, the ligand-dependent activation of PKC primes VEGFR-2 for degradation through its carboxyl-terminal domain involving serine residues 1188 and 1191.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human macrophage colony stimulating factor (rhM-CSF-1) and recombinant murine vascular endothelial growth factor A-164 (rmVEGF-A₁₆₄) were purchased from R&D Systems (Minneapolis, MN). Recombinant human EGF (rhEGF) was purchased from Invitrogen (Carlsbad, CA). The following compounds were purchased from Calbiochem (La Jolla, CA): 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), the potent PKC inhibitor; bisindolylmaleimide I (GF109203X; GFX), the potent γ -secretase inhibitors; Compound E and L-685, 458, and the proteasome inhibitor, MG-132. Mouse monoclonal anti-phosphotyrosine antibodies, PY-20 and 4G10 (IgG_{2b}), were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), respectively. Mouse monoclonal antibody to ubiquitylated proteins (clone FK2; IgG1) was purchased from BIOMOL International (Plymouth Meeting, PA). The rabbit polyclonal antibodies were used as follows: anti-phospho-MAPK (pT202/pY204) and anti-phospho-VEGFR-2 (pY1054/pY1059; Cell Signaling Technology, Beverly, MA); anti-c-Cbl (C-15; sc-170); anti PLC- γ 1 (1249; sc-81); anti-EGFR (EGFR 1005; sc-03); anti-CSF-1 receptor antiserum raised against the carboxy terminus (sc-692; Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit polyclonal anti-VEGFR-2 sera were raised against either a glutathione S-transferase-VEGFR-2 kinase insert domain fusion protein (1410) or a glutathione S-transferase-VEGFR-2 carboxyl-terminus fusion protein (1412; Rahimi *et al.*, 2000; Meyer *et al.*, 2004). Preadsorbed goat anti-rabbit IgG (sc-2054) and goat anti-mouse IgG (sc-2055) secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology.

Cell Lines

Porcine aortic endothelial (PAE) cells ectopically expressing human EGFR, CKR, F1006/CKR, F1173/CKR, R866/CKR, Δ CKR/212, wild-type VEGFR-2, Δ VEGFR-2/152, Δ VEGFR-2/157, Δ VEGFR-2/212, 70Z/3-Cbl, and wild-type human c-Cbl were established by retroviral transduction as described previously (Meyer *et al.*, 2003). Briefly, cDNAs encoding human EGFR (ErbB-1), CKR, F1006/CKR, F1173/CKR, and R866/CKR were cloned into the pLNCX² retroviral expression vector containing a G418 resistance cassette and transfected into 293-GPG cells. cDNAs encoding wild-type VEGFR-2, Δ VEGFR-2/152, Δ VEGFR-2/157, and Δ VEGFR-2/212 were cloned into the retroviral expression vector, pLXSN², and transfected into 293-GPG cells. cDNAs encoding 70Z/3-Cbl and wild-type human c-Cbl were cloned into the pMSCV retroviral expression vector containing a puromycin resistance cassette (Clontech) and transfected into 293-GPG cells. Viral supernatants were collected for 7 d, concentrated by centrifugation, and used as described previously (Meyer *et al.*, 2003).

Site-directed Mutagenesis

Creation of point mutations at codons 866 (Rahimi *et al.*, 2000), 1173, and 1006 (Meyer *et al.*, 2003) within the cytoplasmic domain of murine VEGFR-2 (Fik-1) were described previously. The VEGFR-2 chimera (CKR) was used as a template to construct the carboxyl terminal truncated CKRs (Δ CKR/152, Δ CKR/157, and Δ CKR/212) as described previously (Rahimi *et al.*, 2000; Meyer *et al.*, 2004). Retroviral pLXSN² constructs containing either cDNA inserts encoding each of the truncated CKR receptors or full-length VEGFR-2 were sequentially digested with *NotI* and *Clal* to release either a cDNA fragment encoding the ectodomain domain of the human CSF-1R or VEGFR-2, respectively. The *NotI/Clal* fragment encoding the VEGFR-2

ectodomain was ligated into pLXSN² constructs containing the *Clal/Sall* cDNA fragment encoding the transmembrane and truncated cytoplasmic domains of VEGFR-2. The resulting pLXSN² constructs were named Δ VEGFR-2/152, Δ VEGFR-2/157, and Δ VEGFR-2/212 and were characterized by restriction mapping and nucleotide sequence analysis.

The truncated VEGFR-2 chimera, Δ CKR/157, was used as a template to construct Δ CKR/165/4A, Δ CKR/165/2A, Δ CKR/165/1A, and Δ CKR/196. The carboxyl-terminal domain truncations were made using PCR-based site-directed mutagenesis. Briefly, to create Δ CKR/165/4A, Δ CKR/165/2A, and Δ CKR/165/1A PCR's were performed using a 5'-oligonucleotide containing a *Clal* site corresponding to the engineered *Clal* site in the cDNA of the transmembrane region of CKR (Rahimi *et al.*, 2000) and a 3'-oligonucleotide containing a *Sall* site, a stop codon corresponding to E1202 in the carboxyl-terminal domain of murine VEGFR-2, and the appropriate S/T->A mutations corresponding to S1191, T1194, S1195, and S1198 (Δ CKR/165/4A); S1188 and S1191 (Δ CKR/165/2A); and S1191 (Δ CKR/165/1A) in the carboxyl-terminal domain of murine VEGFR-2. To create Δ CKR/196 PCR's were performed using the *Clal* sense primer and a 3'-oligonucleotide containing a *Sall* site and a stop codon corresponding to Y1173 in the carboxyl-terminal domain of murine VEGFR-2. PCR products were sequentially digested, cut by *Clal* and *Sall*, and then ligated directly into the pLXSN² retroviral vector containing a 5'-*NotI* to 3'-*Clal* cDNA fragment encoding the extracellular domain of human c-Fms. The resultant constructs containing the indicated point mutations/truncations were verified by sequence analysis. The recombinant retroviral plasmids were then used to generate recombinant retroviral particles to transduce PAE cells as described above.

Immunoprecipitation and Western Blotting

Equal numbers of PAE cells or mouse NIH-3T3 fibroblasts expressing the indicated receptors were grown in 100-mm culture dishes until 80–90% confluent cell monolayers were established in 5% CO₂, 95% air at 37°C in DMEM containing 10% fetal bovine serum (FBS) supplemented with L-glutamine, penicillin, and streptomycin. Thereafter, the cell monolayers were starved overnight in serum-free DMEM in preparation for growth factor or phorbol ester stimulation. After serum starvation, cells were left either resting or appropriately stimulated with 40 ng/ml rhCSF-1, 100 ng/ml rhEGF, 100 ng/ml rmVEGF₁₆₄, or TPA for the indicated periods at 37°C. The final concentration of all recombinant growth factors used was as listed above. TPA was dissolved in dimethyl sulfoxide (DMSO) and used in all experiments at a final concentration of 200 nM. For prolonged TPA treatment, cells were incubated in 200 nM TPA for ~20 h before a second stimulation at 200 nM for 1 h with or without rmVEGF₁₆₄. For inhibition of PKC, cells were preincubated with GF109203X (5 μ M) for 30 min at 37°C before a second incubation in the presence of either rmVEGF₁₆₄ or TPA for the indicated period. Unstimulated cells treated with GFX were lysed at the same time as cells subject to PKC inhibition and growth factor stimulation. For γ -secretase inhibition, cells were pretreated with either L-685, 458 (5 μ M) or Compound E (10 nM) for ~16 h in the presence of 0.1% fetal bovine serum-DMEM before a second incubation in the presence of TPA for the indicated period. Where appropriate, control samples were treated with an equivalent volume of solvent. Cells were washed twice with H/S buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM Na₃VO₄) and lysed in lysis (EB) buffer (10 mM Tris-HCl, 10% glycerol, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM Na₃VO₄, and 20 μ g/ml aprotinin). Where indicated, equal amounts of protein representing equal numbers of cells from total cell lysates were either directly resolved by SDS-PAGE or were immunoprecipitated by using appropriate antibodies before SDS-PAGE and immunoblotting. Immunocomplexes were bound to protein A-Sepharose beads (Sigma, St. Louis, MO) and washed three times with 1.0 ml of EB. Total cell lysates or immunoprecipitates were resolved by SDS-PAGE, and the proteins were transferred from the gel onto PolyScreen PVDF Transfer membrane (PerkinElmer Life Sciences, Boston, MA). After transfer, membranes were prepared for immunoblot analysis by incubation for 60 min in a blocking solution containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mg/ml bovine serum albumin, and 0.05% Tween 20. Membranes were then incubated with the appropriate primary antibodies diluted in blocking solution for another 60 min followed by a series of three washes in Western rinse buffer. To visualize immunoreactive bands, membranes were incubated with HRP-conjugated secondary antibodies, washed, and developed with enhanced chemiluminescence (ECL; PerkinElmer Life Sciences). Where indicated, membranes were stripped by incubating them in a stripping buffer containing 6.25 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol at 50°C for 30 min, washed in Western rinse, and reprobed with the primary antibody of interest.

Metabolic Labeling and Analysis of Receptor Turnover

The ligand-dependent down-regulation of CKR and R866/CKR was evaluated by pulse-chase analysis using a mixture of ³⁵S-labeled L-methionine/L-cysteine. In brief, equal numbers of PAE cells were plated in 10-cm tissue culture dishes containing DMEM supplemented with 10% FBS. Cells were then starved for ~16 h in serum-free DMEM. The medium was removed, and cells were rinsed two times with phosphate-buffered saline before an additional 2 h of starvation at 37°C in L-methionine/L-cysteine free DMEM (Invitrogen) supplemented with L-glutamine (Invitrogen). Cells were pulse-

Figure 1. Ligand-induced tyrosine kinase activity is required for accelerated turnover of chimeric VEGFR-2 (CKR). (A) Equal numbers of PAE cells ectopically expressing CKR were serum-starved in Met/Cys-free DMEM for 2 h and then were pulse-labeled with [35 S]Met/Cys (75 μ Ci/ml) for 3 h. Radiolabeled CKR was then chased to the cell surface for the indicated periods in the absence (top panel) or presence (bottom panel) of CSF-1 (40 ng/ml). At each time point, the cells were lysed, and CKR was immunoprecipitated from total cell lysates with an anti-VEGFR-2 antibody that specifically recognizes the kinase insert domain of VEGFR-2 (1410) and analyzed by 7.5% SDS-PAGE followed by autoradiography. The top and bottom arrows on the left indicate the ~180-kDa mature and ~175-kDa immature forms of CKR, respectively. (B) Turnover kinetics of CKR (top panel) and R866/CKR (bottom panel) were analyzed as described in A except that a ~30-min time point was included during the chase period as a control, corresponding to PAE cells that were not stimulated with ligand but were lysed at the same time as PAE cells stimulated with ligand. (C) The relative amount of radiolabeled CKR protein in 1410 immunoprecipitates from each time point during the chase period were measured by densitometry of autoradiographs using the Image Station (Kodak), and the data are expressed as a percentage of the maximal CKR signal detected throughout the course of the experiment (time 0). The graph represents the average of two independent experiments. (D) Equal numbers of serum-starved PAE cells expressing CKR were left either unstimulated or stimulated for the indicated periods with CSF-1 (40 ng/ml). At each time point, cells were lysed and equal amounts of protein from total cell lysates (TCL) were subjected to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). The results shown are representative of at least three independent experiments. (E) The same membrane was reprobed using an anti-PLC- γ 1 antibody as a control for protein loading.

labeled by supplementing L-methionine/L-cysteine-free DMEM with 75 μ Ci/ml EXPRESS labeling mix ([35 S]methionine/cystine; PerkinElmer Life Sciences) for 3 h at 37°C and then chased with complete DMEM supplemented with 100-fold excess of unlabeled L-methionine and L-cysteine. Cells were either left unstimulated or were stimulated with rhCSF-1 (40 ng/ml) at 37°C for the indicated times during the chase period. Finally, cells were washed with ice-cold H/S buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM Na_2VO_4) and lysed with cold lysis buffer (10 mM Tris-HCl, 10% glycerol, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM PMSF, 2 mM sodium orthovanadate, 20 μ g/ml leupeptin, and 20 μ g/ml aprotinin). Equal amounts of protein from each cell lysate representing equal numbers of cells were immunoprecipitated using an anti-VEGFR-2 antibody (1410), which recognizes the kinase insert of VEGFR-2. Immunocomplexed proteins were resolved on 7.5% SDS-PAGE, and gels were prepared for autoradiography by soaking in a fixing solution followed by incubation in an enhancing solution. Gels were then rinsed with water, dried, and exposed to film (Eastman Kodak Co., Rochester, NY) for 1 h at -70°C. Autoradiographs were quantified using the Kodak 1D Image Analysis Software (Eastman Kodak Co.).

RESULTS

Tyrosine Kinase Activity Is Required for Accelerated Ligand-dependent Down-regulation of VEGFR-2

We previously have reported the creation and biochemical characterization of a chimeric VEGFR-2 termed CKR in which the extracellular domain of the human CSF-1R was fused to the transmembrane and cytoplasmic domains of murine VEGFR-2 (Rahimi *et al.*, 2000). We took this unique approach in order to eliminate potential cross-talk between VEGFR-2 and other VEGFR family members. We initially examined the kinetics of receptor turnover. To this end, PAE cells expressing CKR were subject to pulse-chase analysis using [35 S]-labeled amino acids to radiolabel receptor molecules *in vivo*. The total cellular pool of radiolabeled CKR was analyzed by immunoprecipitation, SDS-PAGE, and autoradiography/fluorography (Figure 1A, top panel). Autoradiographs of the radiolabeled CKR immunoprecipitates revealed that in the absence of ligand, the half-life of cell surface CKR was ~100 min (Figure 1C, $\blacktriangle$). However, when saturating concentrations of CSF-1 (40 ng/ml) were added during the chase period, the mature form of labeled CKR underwent significant turnover between 30 and 60 min of stimulation (Figure 1A, bottom panel). The results show that because of the accelerated rate of mature, labeled CKR turnover in the presence of ligand, its half-life was shortened to ~45 min (Figure 1C, $\blacklozenge$). In addition, we also analyzed unlabeled total cell extracts derived from CKR/PAE cells that were left either unstimulated or stimulated for the indicated periods with CSF-1 by SDS-PAGE and immunoblotting with anti-VEGFR-2 antibodies (Figure 1D). The results parallel those presented in Figure 1A (bottom panel) and demonstrate that cell surface-associated CKR undergoes accelerated degradation in response to ligand. Similar results also were obtained with mouse aortic endothelial cells (MAE) endogenously expressing VEGFR-2 (unpublished data).

To address the requirement of VEGFR-2 catalytic activity for its rapid down-regulation in response to ligand, we used a kinase-dead mutant of CKR, named R866/CKR, which contains an arginine-for-lysine substitution at codon 866. We have previously demonstrated that R866/CKR is defective in terms of its ability to undergo ligand-dependent autophosphorylation *in vivo* and *in vitro* (Rahimi *et al.*, 2000). Here, we analyzed the kinetics of ligand-dependent R866/CKR turnover through pulse-chase analysis. Resting PAE cells expressing CKR or R866/CKR were metabolically labeled for 3 h with [35 S]-L-methionine/cystine and then chased for the indicated periods in the presence of CSF-1 (40 ng/ml). Autoradiographs of the radiolabeled CKR immuno-

Figure 2. c-Cbl associates with CKR in vivo but its overexpression does not enhance either the ubiquitylation or turnover of ligand-activated CKR. (A) CKR/PAE cells were retrovirally transduced with either wild-type human c-Cbl or 70Z/3-Cbl constructs as described in *Materials and Methods*. Total cell lysates from equal numbers of CKR/PAE, CKR/c-Cbl/PAE, and CKR/70Z/3-Cbl/PAE cells were analyzed by 7.5% SDS-PAGE and anti-c-Cbl immunoblotting to demonstrate Cbl overexpression. (B) The same membrane was reprobbed with an anti-PLC- γ 1 antibody as a control for protein loading. (C) Equal numbers of serum-starved CKR/c-Cbl/PAE cells were left either unstimulated or stimulated for the indicated periods with CSF-1 (40 ng/ml). At each time point, the total cellular pool of c-Cbl was immunoprecipitated (IP) from total cell lysates using an anti-c-Cbl antibody. c-Cbl immunoprecipitates were resolved by 7.5% SDS-PAGE followed by anti-VEGFR-2 immunoblotting of the top-half of the membrane using an antibody that specifically recognizes the C-tail of VEGFR-2 (1412). c-Cbl-associated CKR is indicated by the arrow on the right. (D) The bottom half of the same membrane was subjected to anti-phosphotyrosine immunoblotting. The arrow on the right indicates 120-kDa tyrosine-phosphorylated c-Cbl. (E) The immunoblot shown in D was stripped and reprobbed with an anti-c-Cbl antibody to demonstrate equal c-Cbl immunoprecipitation for each time point. C–E results were repeated three times with essentially identical results. (F) Equal numbers of PAE cells expressing wild-type CKR (top), CKR/c-Cbl (middle), and CKR/70Z/3-Cbl (bottom) were subjected to pulse-chase analysis as described in *Materials and Methods*. At each time point during the chase period, the cells were lysed, CKR was immunoprecipitated from total cell lysates with an anti-VEGFR-2 antibody (1410), and immunoprecipitates were analyzed by 7.5% SDS-PAGE and autoradiography. (G) The relative amount of radiolabeled CKR protein in 1410 immunoprecipitates from each time point were measured by densitometry using the Image Station (Kodak), and the data are expressed as a

precipitates revealed that the mature, labeled form of CKR underwent significant turnover between 30 and 60 min of stimulation (Figure 1B, top panel). Autoradiographs of the radiolabeled R866/CKR immunoprecipitates, however, revealed that turnover of this mutant receptor was inefficient in response to ligand compared with wild-type CKR (Figure 1B, bottom panel, and 1C, compare diamonds with squares). Indeed, the kinetics of R866/CKR turnover in the presence of ligand appeared to be quite similar to those of CKR in the absence of ligand (Figure 1C, compare squares with triangles). As shown in Figure 1, the kinase-dead CKR (R866) undergoes down-regulation slightly slower than the wild-type CKR with no ligand stimulation, suggesting that basal kinase activity of receptor may contribute to its down-regulation. Collectively, the data presented here clearly demonstrate that selective activation of VEGFR-2 accelerates its rate of degradation and catalytic activity is paramount for its efficient turnover.

Contribution of c-Cbl to Ligand-induced VEGFR-2 Down-regulation

c-Cbl E3-ligase activity is associated with the lysosomal/proteosomal targeting and subsequent degradation of ligand-activated RTKs (Thien and Langdon, 2001). However, the biological role of c-Cbl in VEGFR-2-mediated signal relay in vascular endothelial cells remains to be elucidated. We thus chose to examine the potential role of c-Cbl in mediating ligand-dependent down-regulation of VEGFR-2. For this purpose, we created PAE cell lines overexpressing either wild-type c-Cbl or an E3 ligase-deficient mutant of c-Cbl, 70Z/3-Cbl (Figure 2A). Initial data revealed that in PAE cells, c-Cbl associated with CKR in a ligand-dependent manner (Figure 2C). Anti-phosphotyrosine immunoblotting of the same c-Cbl immunoprecipitates demonstrated a ligand-dependent increase in the tyrosine phosphorylation of c-Cbl. The ligand-dependent tyrosine phosphorylation of c-Cbl and association with CKR appeared to reach a maximum after 15-min stimulation with CSF-1 (Figure 2D). Stripping and reprobing the anti-phosphotyrosine immunoblot with an anti-c-Cbl antibody revealed that equal amounts of c-Cbl protein were immunoprecipitated from each time point (Figure 2E).

To test the effect of c-Cbl on ligand-dependent down-regulation of VEGFR-2, we analyzed receptor turnover as described in Figure 1. Overexpression of either c-Cbl or 70Z/3-Cbl did not alter the ligand-dependent down-regulation of VEGFR-2 (Figure 2, F and G). This was evident on the autoradiographs, particularly after 30- and 60-min stimulation with CSF-1 (Figure 2, F and G). Anti-ubiquitin immunoblotting of CKR immunoprecipitates revealed that neither overexpression of c-Cbl nor 70Z/3-Cbl altered the extent of ligand-induced ubiquitylation of CKR when compared with the level of ubiquitylated receptor observed in the presence of endogenous c-Cbl (Figure 2H). Additionally, anti-phos-

Figure 3. Overexpression of c-Cbl does not enhance VEGF-dependent down-regulation of VEGFR-2. (A) PAE cells expressing wild-type murine VEGFR-2 (Flk-1) were retrovirally transduced with either wild-type human c-Cbl or 70Z/3-Cbl constructs as described in *Materials and Methods*. Total cell lysates from equal numbers of VEGFR-2/PAE, VEGFR-2/c-Cbl/PAE, and VEGFR-2/70Z/3-Cbl/PAE cells were analyzed by 7.5% SDS-PAGE and anti-c-Cbl immunoblotting to demonstrate Cbl overexpression. (B) The same membrane was used for anti-PLC- γ 1 immunoblotting as a control for protein loading. (C) Equal numbers of serum-starved VEGFR-2/PAE cells were left either unstimulated or stimulated for the indicated periods with VEGF-A₁₆₄ (100 ng/ml). At each time point, cells were lysed and equal amounts of protein from total cell lysates (WCL) were subjected to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). (D) The same membrane was used for anti-PLC- γ 1 immunoblotting in order to demonstrate equal protein loading. (E) The kinetics of VEGF-induced down-regulation of VEGFR-2 either in the presence of overexpressed c-Cbl or overexpressed 70Z/3-Cbl was analyzed as in C. (F) The same membrane was used for anti-PLC- γ 1 immunoblotting as a control for protein loading. The results shown in C–F are representative of three separate experiments.

pho-VEGFR-2 (pY1052/pY1057) immunoblotting analyses demonstrated that neither overexpression of wild-type c-Cbl nor 70Z/3-Cbl dramatically reduced or enhanced, respectively, the tyrosine phosphorylation kinetics of VEGFR-2 compared with those observed in the presence of endogenous c-Cbl (unpublished data).

Although we have previously demonstrated that CKR is autophosphorylated in a CSF-1-dependent manner and is able to transduce biological signals in PAE cells, such as mitogenesis (Rahimi *et al.*, 2000), it may be argued that this chimeric receptor does not accurately represent the trafficking patterns of native VEGFR-2. Therefore, to address this issue, we individually overexpressed wild-type c-Cbl and 70Z/3-Cbl at similar levels in PAE cells ectopically expressing wild-type murine VEGFR-2 (Flk-1; Figure 3A). The mature form of wild-type VEGFR-2 underwent rapid ligand-dependent down-regulation with kinetics similar to those

Figure 2 (cont). percentage of the maximal CKR signal detected throughout the course of the experiment (time 0). The graph represents the average of two independent experiments. (H) Equal numbers of CKR/PAE, CKR/Cbl/PAE, and CKR/70Z/PAE cells were serum starved and then left either unstimulated (–) or stimulated (+) for 10 min. with CSF-1 (40 ng/ml). CKR was immunoprecipitated (IP) from (–/+) RIPA cell lysates using an anti-VEGFR-2 antibody (1410) and immunoprecipitates were subjected to 7.5% SDS-PAGE followed by immunoblotting with an anti-ubiquitin antibody to detect ubiquitylated CKR. The bracket on the left indicates the position of ubiquitylated CKR.

Figure 4. Overexpression of c-Cbl enhances EGF-dependent down-regulation of ErbB-1 in PAE cells. (A) PAE cells expressing human ErbB-1 were retrovirally transduced with either wild-type human c-Cbl or 70Z/3-Cbl constructs as described in *Materials and Methods*. Total cell lysates from equal numbers of ErbB-1/PAE, ErbB-1/c-Cbl/PAE, and ErbB-1/70Z/3-Cbl/PAE cells were analyzed by 7.5% SDS-PAGE and anti-c-Cbl immunoblotting to demonstrate Cbl overexpression. (B) The same membrane was used for anti-PLC- γ 1 immunoblotting as a control for protein loading. (C) Equal numbers of serum-starved PAE cells expressing ErbB-1 (lanes 1–6), ErbB-1/c-Cbl (lanes 7–12), and ErbB-1/70Z/3-Cbl (lanes 13–18) were either left unstimulated or stimulated for the indicated periods with EGF (100 ng/ml). At each time point, cells were lysed and equal amounts of protein from total cell lysates (WCL) were subjected to 7.5% SDS-PAGE and anti-EGFR immunoblotting. (D) The same membrane was used for anti-PLC- γ 1 immunoblotting as a control for protein loading. The results presented in C and D is a representative of two independent experiments.

observed for CKR in the context of endogenous c-Cbl as demonstrated by anti-VEGFR-2 immunoblotting (Figure 3C; compare with Figure 1D). Anti-PLC- γ 1 immunoblotting was used as a control for protein loading (Figure 3D). However, anti-VEGFR-2 immunoblotting of total cell lysates derived from cells overexpressing either wild-type c-Cbl or 70Z/3-Cbl demonstrated that the kinetics of VEGFR-2 down-regulation over the indicated periods were not accelerated or delayed, respectively, from those observed in PAE cells expressing endogenous c-Cbl (Figure 3, compare E with C). Anti-phosphotyrosine immunoblotting demonstrated that neither overexpression of wild-type c-Cbl nor 70Z/3-Cbl dramatically reduced or enhanced, respectively, the tyrosine phosphorylation kinetics of VEGFR-2 on the surface of PAE cells compared with those observed in the presence of endogenous c-Cbl (unpublished data).

To assure that the unexpected observation concerning c-Cbl's role in the down-regulation of VEGFR-2 was due to an intrinsic property of the receptor and its trafficking patterns and not to a characteristic of the endocytic system in PAE cells, we overexpressed either wild-type c-Cbl or 70Z/3-Cbl in the context of human ErbB-1 (Figure 4A). We chose to examine the contribution of c-Cbl to ligand-dependent down-regulation of ErbB-1 in PAE cells because these cells lack endogenous EGFRs and ectopically expressed human EGFRs traffic in these cells with kinetics similar to that observed in cells expressing endogenous EGFRs (Carter and Sorkin, 1998). Furthermore, the 175-kDa ErbB-1 is known to be negatively regulated by c-Cbl (Lill *et al.*, 2000). SDS-PAGE and anti-EGFR immunoblotting of total cell lysates derived from ErbB-1/PAE cells that were left either unstimulated or stimulated for the indicated periods with EGF (100 ng/ml) revealed that the mature form of ErbB-1 was subject to pronounced ligand-dependent down-regulation after 60 min of stimulation. The decrease in the electrophoretic mobility of the ErbB-1 immunoreactive band after ligand stimulation was suggestive of receptor ubiquitylation. Virtually all of the receptor underwent down-regulation after 240 min of stimulation with EGF (Figure 4C, lanes 1–6). Anti-EGFR immunoblotting, however, demonstrated that overexpression of wild-type c-Cbl greatly accelerated the rate and extent of mature ErbB-1 down-regulation in response to EGF, with

virtually all of the mature receptor having been degraded after 10 min of EGF stimulation (Figure 4C, compare lane 2 with lane 8). In addition, there was an even more pronounced decrease in the electrophoretic mobility (seen as smearing) of the ErbB-1 immunoreactive band on the gel after 10 min stimulation with EGF, suggestive of enhanced c-Cbl-mediated ubiquitylation. In the context of overexpressed 70Z/3-Cbl, the mature form of ErbB-1 underwent ligand-dependent changes in electrophoretic mobility as well as ligand-dependent down-regulation with kinetics similar to those observed in the context of endogenous c-Cbl as demonstrated by anti-EGFR immunoblotting. However, the magnitude of ErbB-1 down-regulation in the context of overexpressed 70Z/3-Cbl appeared reduced, particularly after 120 and 240 min of EGF stimulation (Figure 4C, compare lanes 1–6 with lanes 13–18).

These results are quite compelling in that in the same PAE cellular context, overexpression of wild-type c-Cbl correlates with an enhanced rate of ErbB-1, but not VEGFR-2, down-regulation although selective activation of VEGFR-2 enhanced the recruitment and tyrosine phosphorylation of c-Cbl (Figure 2, C and D, respectively). These observations also suggest that the observed kinetics of VEGFR-2 down-regulation in the context of endogenous c-Cbl is not due to saturation of a c-Cbl-dependent step by receptor overexpression. In summary, our data shows that c-Cbl does not appear to be the E3 ligase that is responsible for ligand-dependent down-regulation VEGFR-2.

TPA Induces Down-regulation of VEGFR-2

Because we did not observe an accelerated rate of ligand-induced VEGFR-2 turnover in the context of overexpressed wild-type c-Cbl, we wanted to determine if other signaling pathways downstream of VEGFR-2, perhaps involving PKC, play a more pronounced role in ligand-induced down-regulation of VEGFR-2.

TPA belongs to a related class of compounds known as phorbol esters, which are particularly potent and nonmetabolizable PKC activators that mimic the action of diacylglycerol (DAG), a second messenger that is a physiological activator of classical (calcium-dependent) and novel (calcium-independent) PKC isozymes (Newton, 2001). To inves-

Figure 5. TPA induces down-regulation wild-type and kinase-inactive VEGFR-2. (A) Equal numbers of serum-starved PAE cells ectopically expressing wild-type murine VEGFR-2 (Flk-1) were either left unstimulated or stimulated with TPA (200 nM) for the indicated periods. At each time point, cells were lysed and equal amounts of protein from total cell lysates (WCL) were subjected to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). Top and bottom arrows indicate the ~200-kDa mature and ~180-kDa immature forms of VEGFR-2, respectively. (B) The same lysates were subjected to 12% SDS-PAGE and immunoblotting with an anti-phospho-p42/p44-MAPK antibody that recognizes the dually phosphorylated, activated forms of ERK1 and ERK2 indicated by bottom and top arrows, respectively. (C) The same membrane from A was used for anti-PLC-γ1 immunoblotting as a control for protein loading. (D) TPA-induced degradation of R866/CKR in PAE cells was analyzed as described in A. (E) The same membrane was used for anti-PLC-γ1 immunoblotting as a control for protein loading. The results shown are representative of five (A–C) and three (D and E) independent experiments. (F) The relative VEGFR-2/CKR signals were quantified by densitometry using the Kodak Image Station software and are depicted as a proportion of signals observed with unstimulated cell lysates (set as 1).

to investigate a potential role for PKC in modulating the ligand-induced down-regulation of VEGFR-2, we briefly treated PAE cells expressing wild-type murine VEGFR-2 (Flk-1) with TPA (200 nM). Analysis of total cell lysates by SDS-PAGE and anti-VEGFR-2 immunoblotting revealed that cell surface-associated, mature VEGFR-2 was susceptible to TPA-mediated down-regulation with kinetics similar to those observed with VEGF, suggesting that PKC activity may play a role in ligand-induced down-regulation (Figure 5A). Anti-phosphotyrosine immunoblotting revealed that TPA did not alter the tyrosine phosphorylation status of mature VEGFR-2, suggesting that TPA-induced down-regulation of VEGFR-2 was not due to an increase in the intrinsic tyrosine-specific kinase activity of the receptor (unpublished data). The total cellular level of mature VEGFR-2 remained stable in PAE cells treated with vehicle alone (DMSO; unpublished data). Because activation of PKC is known to engage the Raf-1-MEK-MAPK pathway (Sozeri *et al.*, 1992), immunoblotting of the same cell lysates with anti-phospho-MAPK antibodies recognizing the dually phosphorylated (activated) forms of p42/p44 MAPK was included as a control for TPA-induced PKC activity. Figure 5B clearly demonstrates that TPA, through activation of PKC, promoted a robust activation of MAPK. Anti-PLC-γ1 immunoblotting was used as a control for protein loading (Figure 5C).

As demonstrated in Figure 1B, a kinase-inactive VEGFR-2 mutant (R866/CKR) failed to undergo ligand-accelerated receptor turnover. Perhaps certain PKC isozymes, activated downstream of VEGFR-2, are the ultimate mediators of ligand-dependent VEGFR-2 down-regulation and that kinase activity is required for their activation. Thus, we examined if direct activation of PKC by TPA would render R866/CKR susceptible to down-regulation. Anti-VEGFR-2 immunoblotting demonstrated that R866/CKR is capable of undergoing accelerated down-regulation in response to short-term treatment with TPA (Figure 5D). Taken together, these results demonstrate that TPA promotes the turnover of cell surface-associated VEGFR-2. Although it is evident that TPA does

not promote accelerated VEGFR-2 down-regulation through an increase in its intrinsic kinase activity and that TPA can bypass the requirement for kinase activity-induced down-regulation, it remains unclear if ligand- and TPA-induced down-regulation of VEGFR-2 occur through independent mechanisms.

Activation of PKC Is Required for VEGF- and TPA-induced Down-regulation of VEGFR-2 and Occurs Independently of PLC-γ1 Activation

To determine whether endogenous PKC activity also plays a role in VEGF-induced VEGFR-2 down-regulation in PAE cells, we pretreated PAE cells expressing wild-type VEGFR-2 with GFX, a potent inhibitor of PKC (Toullec *et al.*, 1991). Subsequent treatment of VEGFR-2 PAE cells with either VEGF (100 ng/ml) or TPA (200 nM) stimulated significant down-regulation of VEGFR-2 (Figure 6A). The down-regulation of VEGFR-2 in response to TPA stimulation was more pronounced compared with down-regulation induced by VEGF most likely due to a direct and sustained activation of PKC by TPA. However, when cells were pretreated with GFX both VEGF- and TPA-induced VEGFR-2 down-regulation were abolished (Figure 6A). In a second and independent pharmacological approach, we affected PKC inhibition in PAE cells expressing VEGFR-2 by chronic TPA treatment. Prolonged treatment of cells (~20 h) with TPA leads to the depletion of many, but not all, PKC isozymes (Nishizuka, 1992). Depletion of cellular PKC isozymes through chronic TPA treatment abolished VEGF- and TPA-induced down-regulation of VEGFR-2 (unpublished data).

To characterize which class of PKC isozymes are involved in mediating ligand- and TPA-induced VEGFR-2 down-regulation, we used two tyrosine mutant VEGFR-2 chimeras (F1006/CKR and F1173/CKR) fully capable of undergoing ligand-dependent autophosphorylation but defective in their ability to recruit and activate PLC-γ1 (Takahashi *et al.*, 2001; Meyer *et al.*, 2003). To this end, PAE cells individually expressing CKR, F1006/CKR, or F1173/CKR at comparable levels were left

Figure 6. VEGF- and TPA-induced down-regulation of VEGFR-2 requires activation of PKC isozymes independent of PLC- γ 1 activation and does not occur through γ -secretase-dependent cleavage. (A) Equal numbers of serum-starved PAE cells expressing wild-type VEGFR-2 were treated with either DMSO or a PKC inhibitor, GF109203X (GFX; 5 μ M), for 30 min before stimulation with either VEGF-A₁₆₄ (100 ng/ml) or TPA (200 nM) for 60 min. At each time point, cells were lysed, and equal amounts of protein from total cell lysates (WCL) were subjected to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). (B) The same membrane was reprobed with an anti-PLC- γ 1 antibody as a control for protein loading. (C) Equal numbers of serum-starved PAE cells ectopically expressing CKR, F1006/CKR, and F1173/CKR were either left unstimulated or stimulated for the indicated periods with CSF-1 (40 ng/ml). At each time point, cells were lysed and equal amounts of protein from WCL were subjected to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). (D) The same membrane was reprobed with an anti-PLC- γ 1 antibody as a control for protein loading. (E) Equal numbers of PAE cells expressing wild-type murine VEGFR-2 (Flk-1) were pretreated for ~16 h with either DMSO or the γ -secretase inhibitor L-685, 458 (5 μ M). Cells were then left unstimulated or stimulated with TPA (200 nM) for the indicated periods. At each time point, cells were lysed and equal amounts of protein from WCL were subject to 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). (F) The same cell lysates were subject to 12% SDS-PAGE and anti-VEGFR-2 immunoblotting (1412). The positions of the molecular mass markers are indicated with dashes on the left. n.s. indicates a nonspecific band present in immunoblots of total cell lysates with this anti-VEGFR-2 antibody. (G) The same membrane from F was subject to anti-PLC- γ 1 immunoblotting as a control for protein loading. Results presented in A–G are a representation of two independent experiments. The relative VEGFR-2/CKR signals were quantified by densitometry using the Kodak Image Station software and are depicted as a proportion of signals observed with unstimulated cell lysates (set as 1).

either unstimulated or stimulated for the indicated periods with CSF-1 and down-regulation of the receptors was analyzed. As demonstrated in Figure 6C, both F1006/CKR and F1173/CKR are able to undergo down-regulation in response to CSF-1 stimulation with kinetics comparable to that of wild-type CKR. Collectively, the data demonstrate that ligand- and TPA-dependent activation of PKC downstream of VEGFR-2 is required for receptor down-regulation. However, the ability of ligand and TPA to induce VEGFR-2 down-regulation is not regulated by PKC isozymes whose activation is dependent on PLC- γ 1 activity.

PKC-induced Down-regulation of VEGFR-2 Occurs through a Mechanism Independent of Proteolytic Processing

To address the mechanism by which PKC induces down-regulation of VEGFR-2, we first analyzed the putative role of

γ -secretase whose activity has been shown to mediate the intramembrane cleavage of some RTKs including ErbB-4 (Ni *et al.*, 2001) and CSF-1R (Rio *et al.*, 2000; Wilhelmssen and van der Geer, 2004). If PKC-mediated down-regulation of wild-type VEGFR-2 in PAE cells is initiated by metalloprotease-mediated ectodomain shedding at the cell surface, then a membrane-associated ~62-kDa remnant consisting of the transmembrane and cytoplasmic domains should be generated in conjunction with a pronounced decline in mature VEGFR-2 after TPA treatment. Therefore, inhibition of γ -secretase activity in PAE cells expressing wild-type VEGFR-2 would result in accumulation of the VEGFR-2 cytoplasmic domain proteolytic remnant after TPA treatment and allow for its detection. To this end, PAE cells expressing wild-type VEGFR-2 were pretreated with either DMSO as a control or a γ -secretase inhibitor, L-685, 458,

Figure 7. Lack of the VEGFR-2 carboxy terminus impairs TPA-induced down-regulation. (A) Schematic structures of VEGFR-2 and its truncated counterparts possessing progressive deletions of 152 and 212 amino acids are named Δ VEGFR-2/152 and Δ VEGFR-2/212, respectively. The ectodomain swapped Δ VEGFR-2/212 chimera, Δ CKR/212, was previously described (Meyer *et al.*, 2004). (B) Equal numbers of serum-starved PAE cells ectopically expressing wild-type murine VEGFR-2 (Flk-1), Δ VEGFR-2/152, or Δ VEGFR-2/212 were either left unstimulated or stimulated for the indicated periods with TPA (200 nM). At each time point, cells were lysed and equal amounts of protein from total cell lysates (WCL) were analyzed by 7.5% SDS-PAGE and anti-VEGFR-2 immunoblotting (1410). (C) The same membrane was stripped and reprobed with an anti-PLC- γ 1 antibody as a control for protein loading. (D) TPA-induced turnover of CKR (lanes 1–4) and Δ CKR/212 (lanes 5–8) in PAE cells was analyzed as described in B. (E) The same membrane was stripped and reprobed with an anti-PLC- γ 1 antibody as a control for protein loading. Results presented in B–E are a representative of two independent experiments. The relative VEGFR-2/CKR signals were quantified by densitometry using the Kodak Image Station software and are depicted as a proportion of signals observed with unstimulated cell lysates (set as 1).

followed by short-term TPA treatment. As predicted, SDS-PAGE and anti-VEGFR-2 immunoblotting of total cell lysates revealed that mature VEGFR-2 was susceptible to TPA-induced down-regulation independent of γ -secretase inhibition (Figure 6E). Anti-VEGFR-2 immunoblotting of the same lysates using antibodies directed against different cytoplasmic domain epitopes did not reveal an accumulation of ~62-kDa peptide subsequent to γ -secretase inhibition and short-term TPA treatment (Figure 6F). Similar results were obtained with VEGFR-2/PAE cells treated with another γ -secretase inhibitor, compound E (unpublished data). To investigate if this mode of PKC-mediated down-regulation of VEGFR-2 is unique to a PAE cell background, we performed identical experiments with NIH-3T3 fibroblasts ectopically expressing murine VEGFR-2. We demonstrated that short-term TPA treatment is also able to efficiently promote the down-regulation of mature VEGFR-2 in NIH-3T3 fibroblasts without a concomitant increase in a ~62-kDa

proteolytic fragment representing the transmembrane and cytoplasmic domains of the receptor (unpublished data). Furthermore, inhibition of γ -secretase activity did not promote an accumulation of a ~62-kDa fragment in this cell background. Identical experiments were performed in NIH-3T3 fibroblasts ectopically expressing CKR and similar results were obtained (unpublished data). Additionally, TPA-induced down-regulation of mature CKR in PAE cells is blocked upon inhibition of the 26S proteasome (unpublished data). In contrast, brief TPA treatment of NIH-3T3 cells ectopically expressing full-length hCSF-1R resulted in efficient down-regulation of the mature receptor with a concomitant increase in a ~50-kDa proteolytic fragment corresponding to the transmembrane and cytoplasmic domains of the hCSF-1R (unpublished data). The ~50-kDa proteolytic remnant of the hCSF-1R corresponding to its transmembrane and cytoplasmic domains was stabilized upon inhibition of γ -secretase activity. These results strongly suggest

Figure 8. The presence of S1188 and S1191 within the carboxyl-terminal domain of VEGFR-2 is required for efficient ligand-induced down-regulation. (A) Schematic presentation of C-tail truncated chimeric VEGFR-2 (CKRs) in which the carboxyl-terminal domain is deleted by 157, 165, or 196 amino acids. The eight putative S/T phosphorylation sites are denoted numerically according to their position in the primary amino sequence of murine VEGFR-2. Δ CKR/196 retains 15 amino acids in its carboxyl-terminal domain, but unlike Δ CKR/157, the eight remaining S/T residues were deleted. Specific S/T $\rightarrow$ A point mutations were introduced in the context of the Δ 165 truncation as indicated by Δ CKR/165/4A and Δ CKR/165/2A. (B) Equal numbers of serum-starved PAE cells ectopically expressing Δ CKR/157, Δ CKR/165/4A, and Δ CKR/165/2A at comparable levels were left either unstimulated or stimulated for the indicated period with CSF-1 (40 ng/ml). Cells were lysed and whole cell lysates (WCL) were subjected to 7.5% SDS-PAGE followed by anti-VEGFR-2 (1410) immunoblotting. (C) The same lysates from B were also subject to 7.5% SDS-PAGE followed by immunoblotting with an anti-phospho-VEGFR-2 (pY¹⁰⁵²/pY¹⁰⁵⁷) antibody. (D) Immunoblotting with an anti-PLC- γ 1 antibody was used as a control for protein loading. (E) The relative amount of receptor in WCL from each time point was measured by densitometry using the Image Station (Kodak), and the data are expressed as a percentage of the maximal CKR signal detected throughout the course of the experiment (time 0). The graph represents the average of two independent experiments.

that activation of PKC promotes down-regulation of VEGFR-2 through a selective mechanism involving proteasome-mediated degradation that is neither cell-specific nor dependent upon sequential proteolytic processing initiated by metalloproteolytic ectodomain shedding.

The Carboxyl-Terminal Domain of VEGFR-2 Mediates PKC-induced Down-regulation

Because γ -secretase inhibition did not promote a PKC-induced accumulation of a membrane-associated ~62-kDa peptide representing the transmembrane and cytoplasmic domains of VEGFR-2, we chose to investigate the possibility that the cytoplasmic domain of VEGFR-2 may mediate PKC-induced down-regulation. The possibility that initiation of PKC-dependent VEGFR-2 down-regulation is dictated by sequences within its cytoplasmic domain rather than ectodomain sequences was addressed through the creation of a panel of truncated receptors containing progressive carboxyl-terminal domain deletions of 152, 157, and 212 amino acids. These mutant receptors were designated delta VEGFR-2/152, delta VEGFR-2/157, and delta VEGFR-2/212, respectively (Figure 7A). Each truncated receptor was

individually expressed at levels comparable to full-length murine VEGFR-2 (Flk-1) in PAE cells. These carboxyl-terminus deleted receptors permitted us to map locations within the carboxyl terminal domain of VEGFR-2 that may play a role in mediating PKC-controlled down-regulation. Short-term TPA treatment revealed that the mature forms of both full-length VEGFR-2 and delta VEGFR-2/152 underwent significant down-regulation (Figure 7B). Similar results were obtained from identical experiments performed on PAE cells expressing delta VEGFR-2/157 (our unpublished data). These data clearly demonstrate that 157 amino acids of the extreme carboxy terminus of VEGFR-2 are not required for TPA-induced receptor down-regulation. In contrast, TPA treatment of PAE cells expressing delta VEGFR-2/212 demonstrated that the ability of this receptor to undergo down-regulation was severely compromised (Figure 7B).

In a separate approach, we performed ectodomain-swapping experiments in order to highlight a C-tail requirement for PKC-induced down-regulation of VEGFR-2. Specifically, the ectodomain of both full-length VEGFR-2 and delta VEGFR-2/212 was replaced with that of the human CSF-1R (hCSF-1R), thereby creating CKR and delta CKR/212, re-

spectively (Figure 7A). It has been demonstrated that metalloproteolytic shedding of the hCSF-1R ectodomain is the initiating step in PKC-mediated turnover of the hCSF-1R (Downing *et al.*, 1989; Wilhelmsen and van der Geer, 2004). Ectodomain shedding of this receptor is mediated by TACE, a cell surface-associated metalloprotease that recognizes a specific ectodomain sequence (Rovida *et al.*, 2001). Therefore, we predicted that the hCSF-1R ectodomain would confer upon the truncated VEGFR-2 receptor the ability to undergo TPA-mediated down-regulation with kinetics similar to those of mature, full-length VEGFR-2. Although TPA induced the down-regulation of mature CKR, SDS-PAGE and anti-VEGFR-2 immunoblotting of total cell lysates derived from PAE cells expressing delta CKR/212 treated with TPA for 60 min demonstrated that the mature form of this receptor, like its truncated VEGFR-2 counterpart, was resistant to TPA-mediated down-regulation (Figure 7D). Collectively, these data provide additional evidence in support of a mechanism in which activation of PKC initiates down-regulation of VEGFR-2 independent of metalloproteolytic ectodomain shedding. Instead, the PKC-controlled pathway in PAE cells converges upon the carboxyl-terminal domain of VEGFR-2 and primes the receptor for down-regulation.

The presence of 57 amino acids in the carboxyl tail of VEGFR-2 preserves both TPA- and ligand-stimulated down-regulation of VEGFR-2. Within the 57 amino acid stretch in this region there are eight putative serine/threonine phosphorylation sites (Figure 8A). Deletion of 39 amino acids (this truncated receptor called Δ CKR/196) from this region including eight serine/threonine sites impaired the ability of VEGFR-2 to undergo efficient ligand- and TPA-induced down-regulation (unpublished data), suggesting that the presence of these serine/threonine residues may be required for the ability of VEGFR-2 to undergo down-regulation. To address the role of these putative phosphorylation sites on the down-regulation of VEGFR-2, we have created a panel of carboxyl terminus deleted chimeric receptors with the indicated serine/threonine mutations (Figure 8A).

Mutation of serines 1191, 1195, and 1198 and threonine 1194 (Δ CKR/165/4A) on VEGFR-2 had no apparent effect on the ability of VEGFR-2 to undergo down-regulation in response to ligand stimulation. Down-regulation of this mutant receptor was only slightly delayed (Figure 8, B and E). However, a double mutation of serines 1188 and 1191 (Δ CKR/165/2A) greatly reduced the ability of VEGFR-2 to undergo down-regulation (Figure 8, B and E). Indeed, after 30 and 60 min of stimulation with ligand, Δ CKR/165/2A underwent only 25% and 55% down-regulation, respectively, whereas Δ CKR/157 underwent 45% and 80% down-regulation, respectively, under similar experimental conditions (Figure 8E). The rate of down-regulation of Δ CKR/157 is highly similar to that of wild-type CKR (Meyer *et al.*, 2004). The serine/threonine mutated VEGFR-2s were able to undergo ligand-dependent autophosphorylation and activation (Figure 8C). Mutation of serines 1179 and 1183 and threonine 1181 had no effect on the down-regulation of VEGFR-2 (unpublished data). Taken together, these results suggest that the presence of serine1188 plays a major role in regulating the ligand-dependent down-regulation of VEGFR-2. Serine 1191 may also play a minor role in this process.

DISCUSSION

Down-regulation of RTKs is a mechanism known to modulate their signaling. By reducing the receptor level, cells become desensitized to further stimulation by the ligand

(Sorkin *et al.*, 1996). In this report, we have demonstrated that VEGF promotes accelerated down-regulation of VEGFR-2 in a c-Cbl-independent manner and that intrinsic tyrosine kinase activity of VEGFR-2 is required for its ligand-dependent down-regulation. It is believed that c-Cbl is involved in down-regulation of several RTKs including ErbB-1 (Levkowitz *et al.*, 1998, 1999; Waterman *et al.*, 1999; Lill *et al.*, 2000; Jiang *et al.*, 2003) and PDGFR α/β (Miyake *et al.*, 1998, 1999). In this regard, Cbl is considered a rate-limiting component of the endocytic machinery that selectively identifies autophosphorylated forms of these receptors and enhances their degradation through ubiquitylation. In support of these findings, we demonstrated that overexpression of c-Cbl enhances ligand-dependent down-regulation of ErbB-1 in PAE cells. Recently, c-Cbl has also been implicated in the negative regulation of VEGFR-2-controlled biological responses through enhancement of receptor ubiquitylation and degradation (Duval *et al.*, 2003). In this study, however, we were not able to detect alterations to the kinetics of either VEGF-induced VEGFR-2 down-regulation or tyrosine phosphorylation in cells overexpressing wild-type c-Cbl or 70Z/3-Cbl, but demonstrate that c-Cbl is recruited to VEGFR-2 and is phosphorylated on tyrosine residues. Although our findings do not exclude c-Cbl as a component of the cellular machinery that regulates the endosomal sorting of autophosphorylated VEGFR-2 molecules to the 26S proteasome, our data demonstrates that c-Cbl is not a rate-limiting factor in this process. It is possible that the transient association between c-Cbl and VEGFR-2 does not lead to productive ubiquitylation and enhancement in receptor degradation. Indeed, we demonstrate that overexpression of either c-Cbl or 70Z/3-Cbl failed to enhance or diminish ligand-induced ubiquitylation of CKR, respectively. A recent report demonstrated that TGF α , in contrast to EGF, is unable to promote efficient degradation of internalized EGFR because receptor association with c-Cbl and polyubiquitylation are not sustained (Longva *et al.*, 2002). Additionally, structure-function analyses of c-Cbl suggest that its ability to promote EGFR down-regulation is not necessarily linked with its function as an E3 ubiquitin ligase (Yoon *et al.*, 2000; Thien *et al.*, 2001). Thus, the possibility that the E3-ligase activity of c-Cbl is dispensable for its ability to regulate VEGFR-2 down-regulation or that other E3 ubiquitin-protein ligases are involved requires further investigation.

Although the functional roles of the association of Cbl with RTKs are well characterized, the role of Cbl interactions with other components of RTK signaling pathways has remained less clear. Given the evolutionarily conserved function of Cbl family proteins as ubiquitin ligases, it has been suggested that Cbl may also target non-kinase signaling proteins for ubiquitylation, which in turn may either target these proteins for degradation or regulate their function independently of degradation. Although both negative and positive roles for c-Cbl have been proposed in other RTK systems, the precise biological function of c-Cbl in VEGFR-2 signaling is unclear. In light of c-Cbl's association with VEGFR-2, an important question that requires further investigation is the possibility that c-Cbl negatively regulates VEGFR-2 signaling in a manner independent from enhancement of receptor ubiquitylation and turnover.

The PKC family of serine/threonine kinases also has been shown to play a significant role in the down-regulation of several RTK systems including c-Kit (Yee *et al.*, 1993) and CSF-1R (Downing *et al.*, 1989). TPA-induced activation of PKC promotes down-regulation of these receptors at the cell surface through ectodomain shedding and this mechanism is independent from that utilized by ligand. Our present

study reveals that PKC controls down-regulation of VEGFR-2 in a novel mechanism in which the ectodomain of VEGFR-2 is not involved. Our findings also demonstrate that activation of PKC isozymes involved in the regulation of VEGFR-2 turnover occurs independently of PLC- γ 1 activation, suggesting that classical PKC isozymes are not required to promote VEGFR-2 turnover. More detailed studies are needed to identify the PKC isozyme(s) involved in the regulation of VEGFR-2 down-regulation and the mechanism through which they are activated in response to VEGF.

Most recent studies implicating PKC in the negative regulation of RTKs have focused on its ability to induce receptor degradation through RIP (Ni *et al.*, 2001; Wilhelmsen and van der Geer, 2004). Data presented in this study demonstrate that ligand-induced VEGFR-2 down-regulation through activation of PKC, does not employ a mechanism dependent upon proteolytic processing at the cell surface. We could not exclude the possibility, however, that the transmembrane domain of VEGFR-2 is subject to cleavage by proteases other than γ -secretase. A recent study suggests that the ectodomain of the hCSF-1R is a target for TACE, a PKC-induced metalloproteolytic ectodomain sheddase belonging to the ADAM family of membrane-anchored metalloproteases (Rovida *et al.*, 2001; Wilhelmsen and van der Geer, 2004). Replacement of the ectodomain of VEGFR-2 with that of the hCSF-1R did not rescue the inability of activated PKC to promote down-regulation of delta VEGFR-2/212. It is unclear why the ectodomain of the hCSF-1R is unable to confer upon the truncated receptor susceptibility to TPA-induced down-regulation. However, multiple possibilities may explain this result. First, although it has been shown that the ectodomain of the hCSF-1R can be cleaved in the context of other RTK transmembrane domains (Wilhelmsen and van der Geer, 2004), it is possible that the transmembrane domain of VEGFR-2 prevents cleavage of the hCSF-1R ectodomain. This does not seem likely, since full-length CKR is susceptible to TPA-induced down-regulation. It is also possible that PAE cells do not express metalloproteolytic enzymes involved in ectodomain shedding of cell surface receptors. Finally, the metalloproteolytic phenotype of PAE cells, the topography of the plasma membrane with respect to VEGFR-2 and metalloproteases, and the ectodomain identity may be irrelevant. Rather, modification of the VEGFR-2 cytoplasmic domain may represent a selective mechanism through which activation of PKC targets VEGFR-2 for down-regulation. In support of this possibility, we have demonstrated that full-length VEGFR-2 is efficiently down-regulated independent of proteolytic fragmentation in response to short-term TPA treatment in endothelial as well as fibroblast cells. Furthermore, inhibition of the 26S proteasome blocks TPA-induced down-regulation of mature receptor. We have identified a stretch of 39 amino acids within the VEGFR-2 carboxyl-terminal domain required for PKC to induce VEGFR-2 down-regulation. Indeed, within this sequence of 39 amino acids, there are eight putative serine/threonine phosphorylation sites. Among these residues, serine 1188 of VEGFR-2 plays a major role in ligand-dependent down-regulation of VEGFR-2. Thus, it is highly likely that PKC may directly or indirectly through another serine/threonine kinase, control phosphorylation of this residue, thereby targeting the receptor for proteasomal degradation. This prospect is novel because PKC-mediated phosphorylation of specific residues within the cytoplasmic domain of EGFR (Cochet *et al.*, 1984; Downward *et al.*, 1985; Lund *et al.*, 1990) and c-Met (Gandino *et al.*, 1994) down-modulates receptor signaling by inhibiting kinase activation rather than through degradation.

Figure 9. Proposed model of ligand-dependent down-regulation of VEGFR-2. VEGF-mediated VEGFR-2 activation leads to stimulation of PKC activity. Activated PKC (nonclassical forms of PKC) phosphorylates VEGFR-2 at its carboxy terminus marking the receptor for internalization and degradation. In addition, direct activation of PKC by TPA leads to down-regulation of VEGFR-2 independent of VEGFR-2 autophosphorylation and activation.

In conclusion, our work provides new insights into the mechanisms controlling the negative regulation VEGFR-2 signaling. We propose a potential mechanism for VEGFR-2 down-regulation involving a negative feedback loop whereby activation of nonclassical PKC isozymes either through ligand-induced activation of VEGFR-2 or TPA directs serine phosphorylation of the carboxyl-terminal domain of VEGFR-2, thereby marking the receptor for internalization and proteasomal degradation (Figure 9). In light of the importance for VEGFR-2 signal transduction relay in the molecular control of angiogenesis, it appears that its carboxyl terminal domain plays a pivotal role in both receptor activation and down-regulation in order to ensure tight control of receptor activity.

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