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Targeting Cyclic Nucleotide Signaling

G Protein–Coupled Receptor Trafficking

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Vesicular Trafficking of Tyrosine Kinase Receptors and Associated Proteins in the Regulation of Signaling and Vascular Function

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Abstract—Receptor tyrosine kinases (RTKs) play a pivotal role in the development and function of the cardiovascular system. Ligand-activated RTKs promote numerous downstream signal transduction pathways that lead to vascular permeability, as well as proliferation, migration, and differentiation of vascular endothelia and smooth muscle cells. Ligand binding also promotes internalization of the activated receptors either to downregulate the signaling via degradation of the ligand/receptor complex or to signal from endosomes. However, the outcomes of receptor internalization via clathrin-dependent or caveolar pathways and trafficking mechanisms are incompletely clarified in vascular systems. Activity modulation through endocytosis and vesicular trafficking significantly impacts downstream targets of RTKs such as endothelial nitric oxide synthase (eNOS) and VE-cadherin. RTKs and their associated targets are also transported to the nucleus, where they may directly impact nuclear signaling. Although the nuclear transport pathways are just beginning to be unraveled, it appears that endocytosis and vesicular trafficking are involved. In this review, we discuss the mechanisms by which activated RTKs and the downstream targets eNOS and VE-cadherin may be internalized and transported to various intracellular compartments. How localization and interacting proteins impact protein function and influence signaling is an important theme, as is the potential for modulating signaling through therapeutic targeting of activated receptors and components of the endocytic machinery. (***Circ Res*****. 2006;98:743-756.)**

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Growth factor receptors that belong to the receptor tyrosine kinase (RTK) family and play important roles in the development and function of the cardiovascular tissue include vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), insulin receptor (IR), and insulin-like growth factor receptor (IGFR). Deregulation of 1 or more of the RTKs is implicated in various diseases, including cardiovascular disorders. Abnormal regulation of EGFR, for

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Plasma Membrane Localization, Routes of Internalization, and Fates of Endocytosed RTKs

Receptors	PM Localization	Pathway of Endocytosis	Promoters of Endocytosis and Degradation	Inhibitors of Endocytosis and Degradation	Fates of Endocytosed Receptor	References
EGFR	Caveolae	Clathrin pathway	AP2, Grb2, Cbl, Eps15, epsin, Grb10	Sprouty-1 and 2, Alix, LRIG-1, Nedd4	Degradation, recycling to PM, nuclear localization	18–21, 23, 75, 79
VEGFR-1	*	Clathrin pathway*	Cbl, CD2AP	*	Degradation	37
VEGFR-2	Caveolae	Clathrin pathway	Cbl, Nedd4	Grb10	Degradation recycling to PM, nuclear localization	57, 65, 75
PDGFR	Caveolae	Clathrin pathway	Cbl, Grb2, Grb10, Shc	LRP-1	Degradation, recycling to PM	15, 75, 81
FGFR	*	Clathrin pathway	Rab5, dynamin 2	*	Degradation, nuclear localization	28, 116
KGFR	*	Clathrin pathway	*	*	Degradation	27
IR	Caveolae	Clathrin pathway	GAP, Nedd4	*	Recycling to PM	29, 30, 71, 75, 96
IGF-IR	Caveolae	Clathrin pathway	Grb2, Grb10, Nedd4, Shc, GAP	*	Degradation	29, 30, 71, 75, 96
HGFR	*	Clathrin pathway	Cbl, Grb2, Eps15, dynamin 1	*	Degradation	139

*No direct evidence, although plasma membrane (PM) localization and proteins that activate or inhibit endocytosis and degradation might be commonly used with the other RTKs.

instance, is pivotal in many solid tumors because of its direct proliferative effect on epithelial cells and indirect proangiogenic effect on endothelial cells via VEGF upregulation and VEGFR activation.[1] In the heart, excess EGFR signaling is associated with cardiac hypertrophy and heart failure.[2] Similarly, crosstalk between ligand-activated FGFR and VEGFR, as well as insulin and insulin-like growth factor signaling through their respective receptors, bring about the proliferation of endothelial cells and are important in angiogenesis.[3–5] In addition to endothelial cells, IR and PDGFR regulate proliferation and relaxation of vascular smooth muscle cell and play key roles in the development of atherosclerosis.[6–8] However, among all of the growth factors, the VEGF family remains the most critically important in the regulation of endothelial cell growth and function.[9] In vascular endothelial cells, cellular responses to VEGF are triggered through 2 structurally related RTKs, namely VEGFR-1 (also called fms-like tyrosine kinase 1 [Flt-1])[10] and VEGFR-2 (also called kinase domain region [KDR] or fetal liver kinase-1 [Flk-1]).[11,12] The signaling cascades triggered by RTKs have been extensively elucidated, and monoclonal antibody and small molecule therapies targeted to specific RTKs are increasingly being implemented to treat human diseases resulting from receptor hyperactivation. Nevertheless, there remain important gaps in knowledge with respect to RTK function and activation that are centered around receptor internalization and trafficking.

Until recently receptor internalization was thought to be solely for downregulation of receptor activity through degradation. However, increasing evidence indicates that endocytosis is tightly regulated by signal transduction and that signal transduction may be sustained and modulated on endosomes.[13–15] Activity modulation through endocytosis and vesicular trafficking also impacts the downstream targets of receptor tyrosine kinases such as endothelial nitric oxide synthase (eNOS) and VE-cadherin, thereby ensuring a concerted response to RTK activation. eNOS internalization is critical for its activation, and enhanced, localized NO production plays multiple important regulatory roles in maintaining vascular homeostasis. Activation of RTKs also results in the dissolution of endothelial cell–specific adhesion through endocytosis of VE-cadherin, thereby promoting cell migration and vascular permeability. Depending on the cellular cues, internalized RTKs may recycle back to the plasma membrane, recruit signaling proteins and actively signal from endosomes, or be degraded. Localizing receptors and their signaling partners to specialized membrane domains, as well as marking and transporting these molecules along different endocytic pathways, have emerged as central mechanisms for controlling receptor and signaling protein levels, availability, and activation.[14] In this review, we discuss what is known about the trafficking and signaling of RTKs and their interacting proteins in the context of the vascular system. It is important to note, however, that there are likely to be cell type–specific differences in pathways that are beyond the scope of this review. We also briefly discuss the potential of therapeutically manipulating RTK signaling and trafficking pathways to ameliorate human vascular diseases in which these pathways play a central role in acute injury or disease progression.

Endocytic Trafficking Pathways: Routes and Mechanisms

Transmembrane receptors, including most RTKs, are predominantly localized in specialized regions of the plasma membrane called lipid rafts, which may or may not be associated with caveolins. Signaling and adapter proteins are also associated with lipid rafts to facilitate signaling from these domains (Table). In addition to signal transduction, ligand activation of RTKs promotes internalization of the activated receptors via clathrin-dependent or -independent pathways (Figure 1).[16] Clathrin-dependent endocytosis is known to occur outside the lipid rafts in the bulk plasma membrane, necessitating that activated RTKs (including EGFR, PDGFR, IR, and IGF-1R) must exit from lipid raft–signaling platforms to be internalized via the clathrin-mediated pathway. However, new studies on EGFR suggest lipid rafts may also coordinate the assembly of nascent

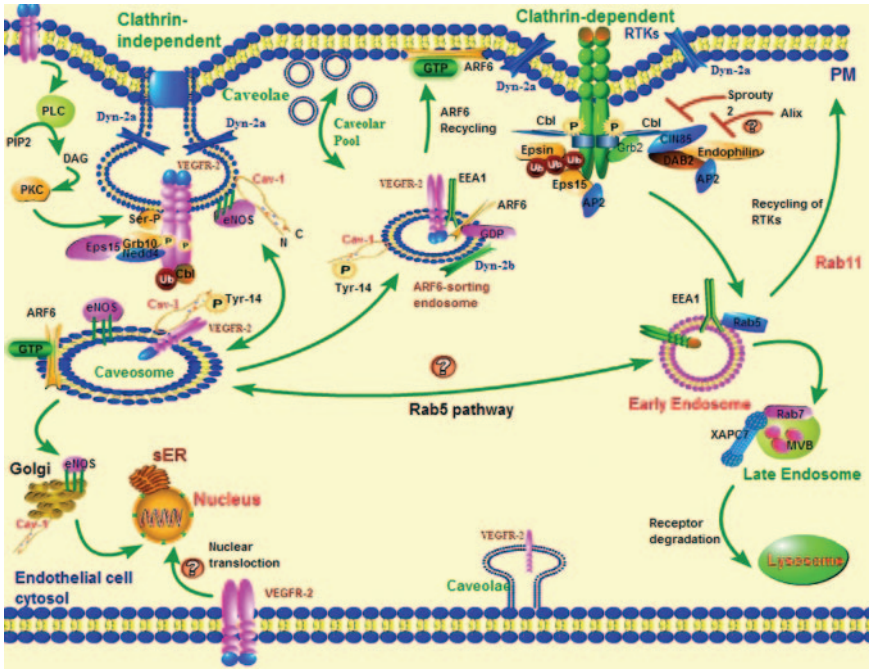

Figure 1. Endocytic pathways and regulatory factors governing vesicular trafficking of RTK(s). RTKs are internalized via 2 major pathways: a clathrin-dependent pathway and a caveolar pathway. Clathrin-dependent uptake involves the recruitment of numerous vesicular trafficking molecules and de novo formation of clathrin-coated membrane invaginations around the activated RTK. The multiadapter protein Grb2 and the ubiquitin ligase c-Cbl bind to the activated RTK and provide further docking sites for CIN85, Eps15, and epsin. These adapter proteins, in turn, recruit clathrin interacting proteins, such as DAB2 and AP2 as well as endophilin and dynamin-2, that facilitate CCV budding and release (not shown). Following internalization, the clathrin coat is shed and the vesicles fuse with Rab5-positive early endosomes. From early endosomes, receptors may recycle back to the plasma membrane or be sorted to late endosomes for lysosomal and/or proteasomal degradation. Proteins like Sprouty 2 and Alix serve as positive regulators of RTK activity by inhibiting their degradation. The caveolar pathway of endocytosis is less characterized and may be used by VEGFR-2. Caveolin-1 serves as the coat protein, and dynamin-2a promotes vesicle budding. Activated receptors are first released from immobile, actin-tethered caveolar invaginations and internalized via small transport vesicles called cavicles (not shown). Adapter proteins such as Grb10 and Eps15 and the ubiquitin ligase Nedd4 may play role in the internalization process. The cavicles are the principal shuttle transporting cargo bidirectionally along microtubules to and from pericentrosomally located caveosomes that are devoid of classic endosomal markers. Molecules internalized via the caveolar pathway may be targeted to the Golgi and the nucleus for signaling or the caveosomes may fuse with the classic endocytic pathway in a Rab5-dependent manner for lysosomal or proteasomal degradation.

clathrin-coated vesicles (CCVs) for the internalization of activated receptors, offering seamless integration of signaling and internalization activities.[17]

Clathrin-Mediated Endocytosis and Signaling

Clathrin-dependent internalization begins with the recruitment of soluble clathrin onto the plasma membrane around the activated RTK and formation of clathrin-coated membrane invaginations, clathrin-coated pits (CCPs), through a series of highly regulated steps. CCPs then pinch off from the plasma membrane to form CCVs. Most RTKs follow this route (see Table). Among RTKs, the endocytic trafficking of EGFR has been studied in the greatest mechanistic detail and serves as a useful paradigm when elucidating the pathways for other RTKs (Figure 1).[18] Activated EGFR recruits the SH2 domain–containing signaling molecule Grb2 (Growth factor receptor–binding protein 2) that, in turn, mediates the binding of EGFR to the E3 ubiquitin ligase c-Cbl to start the cascade of events leading to internalization and degradation.[19,20] Cbl-interacting protein of 85k (CIN85) may then be recruited to disabled2 (DAB2) and endophilin and drive clathrin assembly and budding.[21,22] Ubiquitylation by c-Cbl is an important reversible modification that generates further docking sites for endocytic adapter proteins with ubiquitin interacting motifs (UIMs), such as the adaptor proteins EGFR pathway substrate-15 (Eps15) and epsin.[16] Bridging to clathrin is, in turn, mediated by the clathrin adaptor protein-2 (AP2) or epsin.[23] Thus, the initial assemblies of endocytic machinery components at the plasma membrane are triggered in parallel with EGFR signaling and facilitate formation of nascent coated pits.[17] In this regard, it is noteworthy that EGFR/ErbB2 heterodimerization prolongs plasma membrane signaling after ligand activation by impairing clathrin assembly, most likely in an hsp90-dependent manner.[24,25] Thus, based on the EGFR/ErbB family of receptor paradigm, there is a close coupling between RTK signaling and membrane trafficking.

Analogous to EGFR, other RTKs, such as PDGFR,[26] keratinocyte growth factor receptor (KGFR),[27] FGFR,[28] IR,[29] and IGF-1R,[30] when activated by their respective ligands, are also internalized via the clathrin-mediated pathway (Table). Some of the proteins involved in clathrin-mediated endocytosis, such as Cbl, Grb, and Shc, are shared by most of these receptors, although some may use different signaling and adaptor proteins than EGFR. For instance, clathrin-mediated endocytosis of KGFR is not mediated by Eps15.[27] Differences in adaptor proteins are likely to be important for scaffolding signaling molecules and defining intracellular

fates such as transport to the nucleus, recycling, or degradation. For a number of key receptors in the vascular system, these important details of membrane trafficking remain to be elucidated.

Following internalization, CCVs bearing EGFR shed the clathrin and deliver their cargo to early endosomes (Figure 1). Early endosome delivery is regulated by the Rab5 GTPase, whose activity is, itself, subject to regulation by EGFR signaling.[31,32] Bifurcation of RTK trafficking pathways occurs in early endosomes, allowing for recycling or degradation. Endocytosed RTKs destined for degradation remain ubiquitylated are sorted into luminal vesicles of multivesicular bodies (MVBs) and targeted to lysosomes for degradation by acid-dependent proteases. MVB sorting depends on clustering of cargo within specific phosphoinositide- and clathrin-containing membrane domains. Such cargo clustering requires ubiquitin signal recognition by a large protein complex consisting of HRS (Hepatocyte growth factor–Regulated tyrosine kinase Substrate), TSG101 (Tumor Susceptibility Gene-101), and ESCRT (Endosomal Sorting Complex Required for Transport) proteins that are recruited through specific phosphoinositide- and ubiquitin-binding domains.[16] The ubiquitin signals used in MVB sorting require active c-Cbl.[16,18] Hence, c-Cbl function has been implicated for both internalization and late endosomal sorting, which finally leads toward degradation.

Alternatively, a subset of endocytosed RTKs may recycle back to the plasma membrane, remain associated with and actively signal from endosomes, or even translocate into the nucleus (discussed below in "Nuclear Translocation of RTKs: Mechanisms and Functions?"). Internalized receptors generally recycle from early endosomes back to the plasma membrane in a Rab11-dependent manner.[33] Recycling of EGFR/ErbB2 heterodimers, for instance, is promoted by ligand dissociation in the mildly acidic early endosomes and loss of the ubiquitin signal.[18] Active recruitment of various adaptor and signaling proteins such as Grb2, Shc, phospholipase C (PLC)-γ1, and phosphatidylinositol 3-kinase (PI3K) by activated EGFR and PDGFR on endosomes indicate that ligand-bound RTKs can actively recruit proteins and signal after internalization.[15,34] Furthermore, EGFR-signaling scaffolds continue to be remodeled even en route to degradation with specific mitogen-activated protein kinase (MAPK)-scaffolding proteins and nuclear-signaling proteins recruited to select only endosomes.[35,36]

Like EGFR and PDGFR, activated VEGFR-1 forms a ternary complex with Cbl and a CIN85-related adaptor protein CD2AP (CD2-associated protein), resulting in rapid internalization and degradation.[37] Although the domain harboring inactive VEGFR-1 (caveolae, raft, or clathrin) and serving to link to clathrin via AP2 have not been established, the presence of an AP2 engagement motif in CD2AP[38] suggests ligand-activated VEGFR-1 may also follow the clathrin-mediated pathway. However, in glomerular endothelia, CD2AP has been colocalized with caveolin-1, necessitating further analysis as to its mode of internalization and whether or not VEGFR-1 may also be colocalized with VEGFR-2 in caveolae.[39] CD2AP interacts with the early endosomal Rab4 GTPase, as well as with Cbl and has been shown to regulate the delivery to Rab7-positive late endosomes,[40] suggesting endosomal delivery and lysosomal degradation of VEGFR-1, irrespective of the route of entry. As VEGFR-1 has a 10-fold higher affinity for VEGF than VEGFR-2 and elicits much lower signaling as compared with VEGFR-2, it may predominantly serve to deplete VEGF through endocytosis on the clathrin pathway and degradation in lysosomes.

Caveolar Endocytosis and Signaling

Caveolae are a subset of lipid raft domains seen as morphologically distinct, 50- to 100-nm flask-shaped invaginations that are particularly abundant and critical in the cardiovascular system. In vascular endothelial cells, caveolae represent ≈95% of cell surface vesicles and have multiple functions in organizing and regulating signaling cascades,[41] controlling cell motility,[42] and serving as endocytic carriers.[16,43,44] Caveolae are prevalent on both luminal and abluminal endothelial cell surfaces.[45] On the luminal side, they are poised to serve as highly regulated endocytic carriers for the uptake and transcytosis of luminal albumin and as sensors of shear stress.[43] On both the luminal and abluminal surfaces, they likely function in growth factor and G-coupled receptor signaling and endocytosis. Caveolar structure and function in both signaling and endocytosis depend on caveolin-1 and cholesterol. Caveolin-1 serves to bind and cluster cholesterol. Because of its suggested insertion as a hairpin loop with exposed cytoplasmic N and C termini, and a capacity to oligomerize, caveolin-1 also acts as a coat protein.[46] In addition to caveolin coats, endothelial caveolae have a cohort of associated molecular trafficking machinery. Additional vesicle transport proteins (SNAREs, NSF, SNAP, annexins, monomeric, and trimeric GTPases) may contribute to docking and fusion functions.[47] Unlike the clathrin-dependent pathway, clathrin-independent endocytosis, particularly the caveolar pathway, is a more recently appreciated process and is still being characterized.[43]

Like clathrin-dependent endocytosis, caveolar endocytosis is highly regulated and integrated with active intracellular signaling. In the unstimulated state, caveolae are static structures and are anchored to the actin cytoskeleton.[48] Following receptor stimulation and actin remodeling via the Rho family of small GTPases, caveolar budding occurs and leads to the formation of enclosed intracellular vesicles called "cavicles."[42] Phosphorylation of caveolin-1 (Y14) and dynamin-2 (Y597) by Src kinase and serine/threonine dephosphorylation are required for caveolar fission.[43,49] Interestingly, 2 distinct isoforms of dynamin-2 regulate fission and possibly downstream interactions between caveosomes (see below) and endosomes.[50–52] Elegant new studies reveal that at least 6 kinases regulate caveolin-1 coat assembly, caveolar dynamics, and endocytosis.[53,54] The "cavicles" move their cargo rapidly and bidirectionally on microtubules between the plasma membrane and pericentrosomal static caveosomes.[48,54] Caveosomes are pH neutral and lack all classic markers of endosomes. From caveosomes, internalized cargo may be delivered to the Golgi or the endoplasmic reticulum (ER) via distinct pathways, possibly also providing a retrograde transport pathway to the nucleus for caveolin and associated cargo (Figure 1).[41,54,55] In addition, proteins des-

tined for degradation in lysosomes may be delivered to Rab5-positive endosomes using a "kiss and run" fusion mechanism that avoids dissociation of the caveolin coat.[44] In the case of serine/threonine kinase TGF-β receptor, caveolar uptake can occur in parallel with clathrin-mediated receptor internalization and serves to alter receptor fate and signaling.[56] Although such shunting between clathrin and caveolar pathways has not been described for RTKs, the close association of lipid rafts and de novo clathrin assembly and contributions of receptor heterodimerization to selective trafficking[17,24] suggests that this issue must be analyzed carefully for each receptor. In sum, caveolar endocytosis provides a unique mechanism, distinct from the clathrin pathway, for the integration of receptor signaling, degradation, and possibly nuclear communication via the ER.

Among RTKs, VEGFR-2 may be uniquely internalized preferentially via a caveolar pathway and transported to perinuclear caveosomes (Figure 1).[16,26,57,58] This postulate is predicated on the fact that VEGFR-2 is significantly enriched in plasma membrane caveolae and is absent from CCPs.[55] Furthermore, recent findings report VEGFR-2 colocalized with caveolin-1 in an intracellular perinuclear organelle reminiscent of caveosomes.[50] Additional evidence for the involvement of a clathrin-independent or nonconventional clathrin pathway derives from the observation that activated abluminal VEGFR-2 may exit caveolae and associate with Arf6 and Rac1 in focal adhesions.[59] Arf6 is known to promote plasma membrane recycling and coordinate actin remodeling.[60] Arf6 coordinates uptake of proteins via a clathrin-independent route, although it may also be important in regulating convergence with the clathrin-dependent route and has been shown to bind the clathrin adapter AP2 in vitro.[61,62] Caveolar and/or Arf6-dependent uptake of VEGFR-2 could imply the segregation of VEGFR-1 and VEGFR-2 homodimers into discrete plasma membrane domains and internalization of the 2 receptors by independent pathways. If homodimers of VEGFR-1 and VEGFR-2 prove to be internalized via different pathways, it raises the question of what happens to VEGFR-1 and VEGFR-2 heterodimers that have been shown to result in the activation of discrete signaling cascades in endothelial cells.[63]

Most studies on VEGFR-2 internalization have focused on factors regulating receptor ubiquitylation with degradation used as a read-out assay and do not distinguish among caveolar, Arf6, or conventional clathrin-dependent routes. Two groups found VEGFR-2 ubiquitylated by c-Cbl and colocalization of both proteins.[64,65] A third study failed to find support for a significant role of c-Cbl in VEGFR-2 internalization; rather, a protein kinase C (PKC)-regulated pathway was suggested to be of primary import for regulating internalization.[66] Despite the disagreement about the involvement and role of c-Cbl, there is a general consensus that VEGFR-2 undergoes ligand-stimulated downregulation. Receptor autophosphorylation and ubiquitylation are also crucial in the process. Because c-Cbl has been found in caveolar fractions, as well as in CCVs, its association with VEGFR-2 cannot be used to discriminate clathrin-dependent or caveolar routes of internalization. Therefore, the central question of whether or not VEGFR-2 uses a caveolar route for internalization cannot be unambiguously answered based on available data. Temporal imaging studies modeled on those performed to study caveolar dynamics[54] and TGF-β endocytosis along a caveolar pathway[56] will be required to further resolve this question. Specific pharmacological inhibitors of AP2-mediated clathrin-coat assembly may also be used.[67] In addition, the specific role of Arf6 in VEGFR-2 internalization needs examination. Ultimately, if VEGFR-2 proves to be preferentially internalized in caveolae, the internalized receptor may be delivered from caveosomes to endosomes to enable receptor degradation in lysosomes (see Figure 1). Connections to proteasomal degradative paths would be facilitated by transfer to late endosomes where Rab7 has been shown to recruit proteasomes.[68] Alternatively, receptor recycling may be facilitated by Arf6-regulated endocytic circuits, and/or the receptor may reach the nucleus by as yet uncharacterized routes.[60] Thus, there is impetus for clarifying the VEGFR trafficking pathways, functions, and consequences, as they are likely to differ from those detailed for EGFR and may serve as novel targets for therapeutic intervention and modulation of VEGFR activities.

Regulation of RTK Trafficking by Ligand

RTK trafficking fates are fine tuned by the ligand type, concentration of the ligand, and receptor heterodimerization. For example, the constitutive degradation rate of the EGFR is slower than that of other ErbB family members, and EGFR trafficking is altered in response to ligand binding. Following ligand activation, EGFR gets internalized and signals from endosomes, whereas signaling by other members of the ErbB family members occurs predominantly from the cell surface. Overexpression of ErbB2 slows the internalization of the EGFR and enhances recycling, because of heterodimerization of the receptors. The choice of pathway is also dependent on ligand concentration and, in some cases, ligand type. EGFR, when stimulated with low doses (0.25 nmol/L) of EGF, is internalized almost exclusively through the clathrin-dependent pathway that involves EEA1-, Rab11-, and CD63-positive endosomes. At higher concentrations of EGF (3 nmol/L), however, a substantial fraction of the receptor is endocytosed through a clathrin-independent, lipid raft–dependent route.[58] Decorin, a soluble proteoglycan, causes a slow and sustained internalization of EGFR, primarily via caveolae-mediated endocytosis, and targets the receptor to a distinct intracellular compartment, most likely the "caveosome."[69] Decorin-induced internalization precludes EGFR phosphorylation and does not appreciably involve EEA1-positive early or Rab11-positive recycling endosomes. However, EGFR internalized in a decorin-mediated fashion eventually merges with CD63-positive late endosomes and lysosomes, which supports a convergence of late stages of the clathrin- and caveolin-mediated pathways under some circumstances.

Regulation of RTK Trafficking by Ubiquitylation

One of the signals for internalization and endosomal sorting of RTKs is the covalent addition of ubiquitin by a family of E3 ubiquitin ligases.[70] The importance of 1 of the ubiquitin

ligases, c-Cbl, in orchestrating a cascade of events leading to internalization and degradation of activated RTKs has been outlined above for the clathrin-mediated pathway. However, regulation of membrane transport through ubiquitylation is a complex process that involves different ubiquitin ligases and several positive and negative regulators and is not limited to the clathrin-mediated pathway. As detailed below for EGFR and VEGFR-2, the consequences of ubiquitylation may significantly vary from 1 receptor to the other. In this section, we look at some of the most important regulators of ubiquitylation and their effects on the fate of RTK trafficking.

Nedd4 (Neural precursor cell expressed developmentally downregulated 4), like c-Cbl, is an E3 ubiquitin ligase with both positive and negative roles in endocytosis. It may directly ubiquitylate plasma membrane receptors, such as IGF1-R, and stimulate a clathrin-dependent internalization and lysosomal degradation.[71] Nedd4 also enhances ligand-stimulated VEGFR-2 degradation, although neither a direct Nedd4/VEGFR-2 complex nor Nedd4-mediated VEGFR-2 ubiquitylation was reported,[65] suggesting an indirect role in stimulating VEGFR-2 degradation. In response to EGF, Nedd4 ubiquitylates endocytic proteins (all isoforms Cbl, Eps15, and Hrs) and targets them for proteasomal degradation, thereby prolonging the longevity of EGFR signaling as opposed to stimulating receptor degradation.[72,73] Because Nedd4 has been associated specifically with the apical plasma membrane of epithelial cells, it is important to establish whether Nedd4 exhibits a polarized expression in endothelia.[74] If Nedd4 proves to be restricted to the luminal endothelial surface, its specific role in clearance of luminal versus abluminal VEGFR-2 and other RTKs will need to be carefully considered in an appropriate endothelial cell system.

Nedd4-mediated regulation may be further modulated by the adapter protein Grb10. Grb10 is a multidomain protein that is recruited to tyrosine-phosphorylated RTK via its SH2 (Src Homology domain 2) and BPS (Between-Plekstrin-Homology) domains. Receptor-bound Grb10 then mediates the recruitment of Nedd4 and various other proteins and may positively or negatively modulate receptor signaling and trafficking pathways.[75,76] In the case of insulin responses mediated via IGF1-R, Grb10 serves as a bridging protein for the recruitment of Nedd4 and promotes ubiquitylation of the receptor and degradation of IGF-1R.[71] In contrast, Grb10 appears to be a positive regulator of the VEGF-signaling pathway. Grb10 is upregulated in response to VEGF, stimulates tyrosine phosphorylation of VEGFR-2, and functions to inhibit Nedd4-mediated degradation.[65] However, the underlying mechanisms whereby this occurs remain to be further clarified. For example, excess Grb10 may sequester Nedd4 in the cytoplasm or disrupt a possible ternary complex among Nedd4-Grb10-Eps15 and thereby interfere with receptor endocytosis.[65] In this regard, Grb10 may function much like Sprouty-2 does by sequestering Cbl and thereby blocking degradation.[77]

Ubiquitylation-mediated regulation of mammalian RTKs is further modulated by other proteins such as Alix,[78] Sprouty-1 and -2,[79] LRIG1,[80] and LRP1,[81] which are activated by RTKs and play a positive-feedback role to spare activated RTKs from degradation. Sprouty and LRP1, for instance, directly interact with the ubiquitin E3 ligase (c-Cbl) and sequester it away from the activated RTKs, thereby preventing ubiquitylation and targeting of RTKs such as EGFR and PDGFR for degradation.[81,82] Similarly, Alix binds to the CIN85 and endophilins and attenuates the interaction of these proteins with Cbl, leading to decreased EGFR internalization and degradation.[83] Alix may in turn be inactivated by Src, demonstrating the involvement of hierarchical levels of regulation.[78] The myriad of endocytic proteins involved in the downregulation and endocytic sorting of RTKs make it clear that analogous to the intracellular signaling cascades, the membrane trafficking of RTKs is closely regulated and subject to both positive and negative controls, which in turn modulate RTK signaling outcomes.

Another level of regulation is provided by deubiquitylating enzymes such as UBPY and AMSH (Associated Molecule with the SH3 domain of STAM).[84,85] These enzymes play important roles in endosomal trafficking by negatively regulating ubiquitin-dependent lysosomal sorting and degradation. Deubiquitylating enzymes may directly remove ubiquitin from the ubiquitylated cargo and/or regulate the free ubiquitin pool. Enhanced degradation rates of EGFR have been observed following AMSH knockdown.[84] Similarly, depletion of endogenous UBPY by RNA interference results in elevated ubiquitylation and accelerated degradation of EGF-activated EGFR, whereas overexpression of UBPY reduces the ubiquitylation level of EGFR and delayed its degradation.[85] Taken together, these studies demonstrate the importance of ubiquitylation in RTK trafficking and indicate the complex positive and negative regulatory pathways of ubiquitylation on which the fate of activated receptors depends. As more of the specific ubiquitylation cascades are elucidated, these may serve as novel therapeutic targets for modulating RTK signaling and the physiological consequences (cell migration, cell growth, angiogenesis, etc).

Other Regulators of RTK Trafficking

Dynamin-2 is a well-established regulator of both clathrin-mediated and caveolar endocytosis.[52] Rather surprisingly, a dominant negative mutant of dynamin-2 (K44A) was found to increase VEGFR-2 degradation in human umbilical vein endothelial cells (HUVEC).[50] One would have expected the mutant to interfere with VEGFR-2 degradation rather than increase it. An explanation for this result may derive from the recent identification of 2 isoforms of dynamin-2.[52] Only 1 of the 2 isoforms (dynamin-2a) interfered with caveolar uptake from the plasma membrane as a K44A mutant. The second dynamin-2b isoform may instead be involved in the known kiss-and-run interactions between caveosomes and early endosomes. By analogy with the dynamin-2a K44A mutant that blocks plasma membrane vesicle scission, the dynamin-2b K44A mutant may interfere with the release of caveosomes from endosomal interactions. Consequently, an increasing fraction of VEGFR-2 may be delivered to early endosomes and enter the degradative route to late endosomes and lysosomes. The increased degradation of VEGFR-2 was also found to correspond with a significant growth arrest and has been observed to alter eNOS function and endothelial migration in response to VEGF and NO (N. Kang-Decker and V.

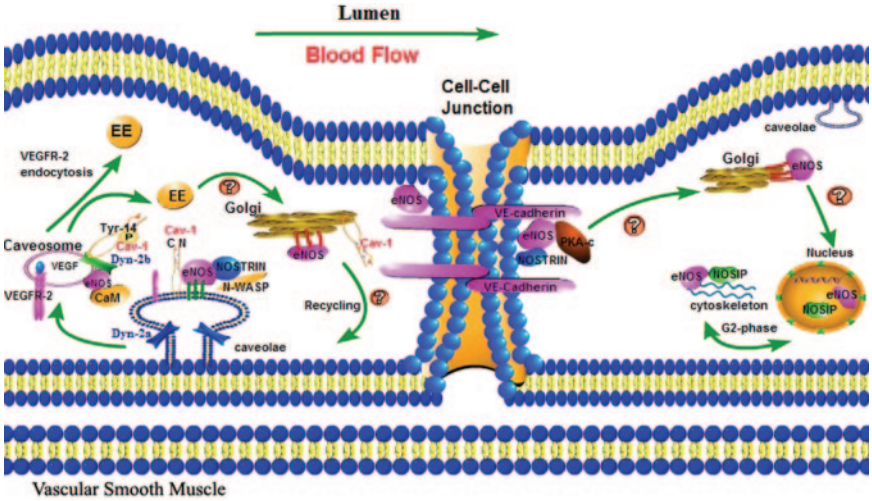

Figure 2. Endocytic trafficking in the subcellular localization and activity of eNOS. eNOS is localized at the cytoplasmic face of plasma membrane caveolae, cell–cell junctions, and the Golgi complex. Inactive eNOS in caveolae is associated with caveolin-1. VEGF stimulation leads to caveolin-1 phosphorylation at Tyr14 and results in the release and activation of eNOS. Caveolar endocytosis and transport to the Golgi complex is suggested to modulate eNOS activity and promote recycling. Plasma membrane–Golgi cycling of eNOS and its functions at the both sites may be regulated by the eNOS interacting proteins NOSIP/NOSTRIN. In the Golgi and at cell junctions, eNOS does not associate with caveolin-1. At cell–cell junctions, basal eNOS activity maintains junctional integrity and may help in responding to mechanostimulation.

Shah, unpublished data, 2005).[86,87] Thus, intracellular signaling and discrete fates of VEGFR-2 may be coordinated through multiple isoforms of dynamin.

In addition to the positive influence on growth factor signals, PKC serine/threonine kinase activation is known to regulate the signaling of RTKs. PKC has been implicated in both endocytic receptor degradation, as exemplified by PDGFR or endocytic recycling in the case of EGFR.[88] In recent work, the C terminus of VEGFR-2 was shown to be phosphorylated by nonclassical PKCs on serines 1188 and 1191, and phosphorylation was required for ligand-stimulated receptor downregulation.[66] Degradation was dependent on proteasome activity. Phorbol ester–mediated activation of PKC, which mimics the action of diacylglycerol, bypassed the need for VEGF and receptor tyrosine phosphorylation for receptor degradation. Together these data suggest an important role for nonclassical PKC in controlling VEGFR-2 activity through receptor downregulation. A number of PKC isoforms have been found to be recruited to caveolae in response to phorbol ester stimulation.[89] It will be of interest to determine whether VEGFR-2–induced internalization via the PKC-stimulated pathway impinges on any of the 4 serine/threonine kinases or Src tyrosine kinase involved in caveolar dynamics or whether it involves an Arf6-regulated or clathrin-dependent pathway.

The available data demonstrate the importance of endocytic trafficking in the regulation of receptor function. Modulation of angiogenic responses controlled by VEGFR-2 may take place in caveolae and caveosomes, whereas signaling cascades triggered by most other RTKs are likely to be modulated in endosomal compartments accessed from CCVs. Overall, careful attention to receptor localization to specific membrane domains, dissection of internalization pathways, and identification of regulatory factors in relevant cell types will be required for a complete understanding of RTKs in vascular biology.

Vesicular Trafficking of eNOS: Importance in Signaling and Regulation of Activity

eNOS is the enzyme that is responsible for the biosynthesis of NO in endothelial and epithelial cells and is coordinately regulated by RTKs.[90,91] NO is 1 of the most important nonpeptide endothelium-derived vasoactive factors that regulates several cardiovascular activities such as vasodilatation, vascular permeability, and angiogenesis.[90] Because NO is an extremely reactive and short-lived signaling molecule, its subcellular distribution is mainly determined by the subcellular localization of eNOS and its local production.[92] A major pool of eNOS resides at the cytosolic face of Golgi complex, with smaller pools in caveolae and endothelial cell junctions, indicating the presence of discrete localizations of eNOS.[41] eNOS is known to redistribute to intracellular locations in response to active RTK signaling, and this relocation may depend in part on active membrane trafficking.[93]

Discrete localizations of eNOS, to caveolae and cell junctions, most likely reflect functionally distinct pools of the enzyme that are regulated by distinct stimuli and endocytic trafficking pathways (Figure 2). Activation of RTKs such as VEGFR, EGFR, and PDGFR induces upregulation of eNOS and NO production.[90] The close coupling between activated VEGFR-2 and eNOS is brought about by their colocalization within caveolae/lipid rafts and direct associations with caveolin-1. The activation of caveolar eNOS depends on caveolin-1 dissociation. In the absence of VEGFR-2 activation, caveolin-1 tightly binds to a motif in the oxygenase domain of eNOS via its special scaffolding domain (amino acids 89 to 95) and maintains eNOS in an inactive state within caveolae.[94] VEGF stimulation leads to Src kinase–mediated phosphorylation of caveolin-1 at tyrosine 14.[57] Consequently, eNOS dissociates from caveolin-1 and becomes activated by binding to calmodulin, dynamin-2a, and several heat shock proteins.[41,86] Thus, caveolin-1 most likely acts as a negative regulator and a scaffold to poise eNOS in close proximity to the machinery controlling localized calcium influx and calmodulin activation, possibly allowing differential eNOS activation at individual subcellular locations.[95] Similarly, activation of the other members of RTKs, namely IR and IGF-1R, in human vascular endothelial cells leads to the phosphorylation and activation of eNOS. However, downregulation of caveolin-1 abolishes the eNOS activation mediated by these 2 (IR and IGF-1R) receptors, indicating that caveolin-1 may serve not only as a negative regulator but also

as a scaffold that positions eNOS in close proximity to the requisite signaling molecules needed for activation.[45,96]

The importance of endocytosis in NO production was first recognized through studies on the bradykinin G protein–coupled receptor.[97] eNOS was found rapidly internalized in a clathrin-independent, but active dynamin-2–dependent, manner, suggesting a caveolar route of uptake.[87] In the absence of initial endocytosis, NO production was blunted. RTK activation, as exemplified by VEGFR-2, can also trigger eNOS endocytosis and activation either in response to ligand or via G protein–coupled receptor crosstalk (Figure 2).[93] In response to ligand, eNOS endocytosis occurs synchronously with caveolin-1 and VEGFR-2 internalization, although it remains unclear whether all 3 proteins traffic together. The eNOS-interacting protein NOSTRIN (eNOS Traffic INducer),[98] dynamin-2, and N-WASP (Neuronal Wiskott–Aldrich Syndrome Protein), a protein controlling actin polymerization, were shown to regulate eNOS internalization in a heterologous cell system.[99] N-WASP is a common downstream target of protein tyrosine kinases including, EGFR, VEGFR, and Src family kinases, with demonstrated importance in vascular physiology.[99–102] Following activation, N-WASP promotes actin polymerization and facilitates RTK endocytosis and endosome motility.[103,104] N-WASP membrane recruitment is highly dependent on the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2),[105] an important substrate in RTK signal transduction and a key regulator of Arf6, as well as of clathrin-dependent pathways.[106] A second eNOS-interacting protein (NOSIP)[107] most likely does not govern membrane transport but rather serves to sequester and inactivate eNOS in a cytoskeletal fraction during the G_2 phase of the cell cycle.[108]

Taken together, the data support a testable model in which endocytosis and trafficking contribute to the rapid and transient activation of eNOS as follows (Figure 2). Immediately after activation, eNOS internalization in cavicles may be mediated by NOSTRIN, N-WASP, and dynamin. Such internalization may maintain eNOS together with VEGFR-2 and dynamin-2 in an activated state, possibly by sequestering them from nonphosphorylated caveolin-1 or inhibitory phosphatases. Once in caveosomes, the VEGFR-2 may be sorted for degradation by transfer to the endocytic pathway, whereas eNOS may be segregated to the Golgi for eventual recycling to the plasma membrane (Figure 2). The carefully orchestrated endocytic transport and activation of eNOS in response to growth factor and/or agonist activation likely serves to restrict cellular responses through localized and transient NO production. Elucidating precisely how this may come about remains a major challenge. The regulation of junctional eNOS and its physiological functions are discussed in "Coordinated Trafficking of RTKs and Adhesion Molecules", together with other RTK-regulated junctional proteins. Thus, membrane-transport pathways could constitute 1 component of a complex regulatory cascade that governs eNOS activity and includes heat shock proteins,[109] posttranslational modification,[110] and the distribution of eNOS between plasma membrane and intracellular pools.

Nuclear Translocation of RTKs: Mechanisms and Functions?

RTKs and associated proteins have frequently been found localized in the nucleus following ligand stimulation, raising the exciting possibility that direct links between RTKs and the nucleus may exist in parallel with signal transduction cascades. Until recently, the mechanisms resulting in nuclear translocation of RTKs remained relatively unexplored. However, the recent characterizations of the nuclear transport of FGFR, EGFR, and VEGFR-2 pave the way for mechanistic and functional analyses of this interesting pathway.[111]

Both ligand-dependent and mechanostimulatory pathways can lead to a rapid (2 minutes) nuclear translocation of VEGFR-2, VEGF, and other associated proteins.[112,113] Transcriptional activation of a reporter under the control of a promoter containing shear-stress response elements (SSREs) and increased accumulation of components of the coagulation cascade were some of the reported consequences.[113] Thus, ligand and/or receptor accumulation in the nucleus may be triggered by both ligand-dependent and -independent stimuli and result in physiologically significant outcomes. The question is: How does the rapid nuclear translocation of VEGFR-2 and other RTKs come about?

There is a significant precedence for a nuclear translocation pathway for RTKs and associated signaling molecules. EGFR, as well as FGFR, has been identified to translocate to the nucleus in a timeframe that precludes retrograde vesicular transport to the ER and delivery to the nuclear membrane by diffusion.[114,115] VEGFR2, like EGFR, appears to accumulate in the nucleus as an intact receptor, and VEGF stimulation was not found to cause metalloprotease-dependent ectodomain shedding or γ-secretase cleavage.[66] The findings leave open critical questions, namely: Is vesicular trafficking involved? In the case of FGFR in epithelia, receptor endocytosis together with E-cadherin is a requisite first step for nuclear translocation.[111,116] Inactivation of endosomal transport by targeting different regulators of this pathway, such as Rab5, dynamin-2, and ARF6, led to a significant reduction in the nuclear localization of FGF. This indicates that endosomal trafficking can play a role in nuclear translocation of RTKs in some cell systems, although parallels in vascular systems remain to be identified. A second open question: Do intact RTKs get released from the plasma membrane or endosomal membrane and, if so, how? Perhaps rapid nuclear transport occurs, not by extensive retrograde vesicular transport but via a process analogous to protein dislocation from the ER in response to improper folding.[117] Distinct AAA ATPases responsible for the local "dislocation" of integral membrane proteins have been found to be associated with mitochondrial and peroxisomal membranes, as well as at prokaryotic cell membranes.[118] Confirmation of this postulate will require the identification of a relevant AAA ATPase and assaying for the dislocation of RTKs from plasma membrane or endosomal fractions using methods similar to those developed for studying ER dislocation.[119] As to the question, What is the role of proteins regulating vascular function in the nucleus? Nuclear translocation of VEGFR-2 is associated with transcriptional activation. However, the observations that nuclear eNOS is largely inactive in the nucleus[95] and that

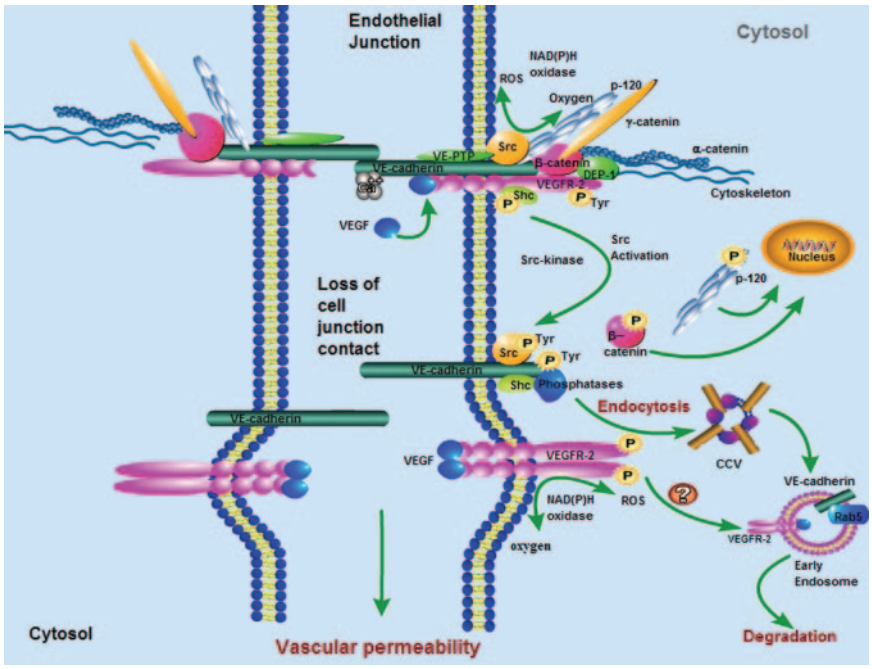

Figure 3. VEGFR-triggered endocytosis of VE-cadherin and loss of endothelial cell–cell adhesion. In intact blood vessels, the extracellular domains of adjacent VE-cadherins associate with each other in a Ca^{2+}-dependent manner to form adherens junctions, whereas the intracellular domain interacts with the cytoskeleton via different members of the catenin family namely, α-, β-, and γ- catenins and p120. VE-cadherin also associates with other regulatory proteins, such as Src kinase and junctional phosphates (DEP-1, VE-PTP, and others). VEGF stimulation leads to phosphorylation of VEGFR-2, Src, and VE-cadherin at the endothelial junctions, which is dependent on NAD(P)H-mediated reactive oxygen species (ROS) generation.[140] At the same time, ROS inhibits VEGFR-2–associated phosphatases to enhance receptor tyrosine autophosphorylation.[141] VEGF stimulation also leads to the phosphorylation and dissociation of β-catenins and p120 from VE-cadherin, enabling their translocation into the nucleus. The dissociation of p120, in particular, results in clathrin-dependent endocytosis and degradation of the VE-cadherin. This promotes loss of cell–cell contacts, vascular permeability, and endothelial cell migration.

the nucleolus maintains proteins in suspended animation[120] would suggest that it may, in some cases, also serve as a stop signal.

Coordinated Trafficking of RTKs and Adhesion Molecules

The vascular endothelium lines the entire cardiovascular system and serves as a selective permeability barrier between the bloodstream and the extravascular space. Extravasation of cells and macromolecules mainly occurs via endothelial cell–cell lateral junctions and is subject to RTK regulation. The integrity of intercellular junctions controls numerous physiological and pathophysiological activities, including angiogenesis, inflammation, hypertension, and arteriosclerosis.[121] VE-cadherin, a member of the calcium-dependent cadherin family expressed in endothelial cells, is the major constituent of adherens junctions. The extracellular domains of VE-cadherin in adjacent endothelial cells form homophilic interactions, and the intracellular domains interact with the actin cytoskeleton via proteins of the armadillo family, β-catenin, plakoglobin (γ-catenin), and p120 (Figure 3).[122] In response to growth factor stimulation, RTKs regulate these interactions between VE-cadherins of adjacent cells and monitor the integrity of the adherens junctions.[122] The interaction of RTKs with adhesion molecules in governing the type and degree of signaling is an emerging paradigm. Complexes of different RTKs with cadherin family members, such as VEGFR with VE-cadherin and EGFR, IGF-1R, and FGFR1 with E-cadherin, have been found in various cell types including endothelial cells.[111,116,123,124] Because the interaction of RTKs with E-cadherin is better studied, we first briefly describe these interactions and then compare what is known and what is missing for VE-cadherin.

In epithelial cells, FGF stimulation leads to cointernalization and colocalization of both FGFR and E-cadherin into Rab5- and EEA1-positive early endosomes. The levels of E-cadherin expression and particularly its localization at cell–cell junctions negatively regulate FGFR1 trafficking and nuclear localization and attenuate FGFR1 signaling.[116] Similarly, interactions between EGFR and E-cadherin or VE-cadherin and VEGFR-2 also limit the mobility and activities of the respective RTKs.[123,125] The E-cadherin/EGFR interaction, in particular, decreases the ligand-binding affinity of EGFR.[125] Taken together, these data imply that the cadherin/RTK interactions lead to either (1) cointernalization of the 2, resulting in loss of cell–cell contact (an effect favoring cell proliferation and migration); or (2) sequestering of the RTKs at the cell–cell junction away from their ligands (a tumor-suppressor function). It is therefore crucial to understand the specific signals leading to 1 or the other of these pathways. To phosphorylate the cadherin and catenins, RTKs at cell junctions have to bypass the neutralizing effects of junctional phosphatases (discussed below). It might be the outcome of the competition between kinases versus phosphatases that decides which of the 2 pathways is activated.

In endothelial cells, VEGF stimulation leads to a complex among VEGFR-2, VE-cadherin, and Src at intercellular junctions and promotes specific tyrosine phosphorylation of VE-cadherin (Y658 and Y731).[126–128] Consequently, the binding of VE-cadherin to p120 and β-catenin is disrupted, and a rapid internalization and degradation of VE-cadherin occurs via a clathrin-dependent pathway (Figure 3).[123,129] The β-catenin and p120 that are released from VE-cadherin may translocate to the nucleus to modulate transcription and cell proliferation. The nuclear translocation of activated RTKs (eg, FGFR) that has been observed after cointernalization with E-cadherin may indicate coordinated nuclear transport with β-catenin and p120. Specific serine and threonine phosphorylation on the NH_2 terminus of p120 is critical for VE-cadherin interaction. Although E-cadherin internalization

in epithelial cells is known to be regulated by ubiquitylation,[130] no such modification or relevant ubiquitin ligase has been identified for VE-cadherin. In addition to the tyrosine kinases and the adhesion molecules, cell junctions also contain several junctional phosphatases that may dephosphorylate the activated RTK and/or its substrates and enable return to an adhesive state. The transmembrane protein VE-PTP is 1 such phosphatase that interacts with and reduces the phosphorylation VE-cadherin.[131] Other junctional phosphatases that interact with phosphorylated VE-cadherin via the Shc adapter protein and function as negative regulators of VEGFR-2–mediated loss of cell–cell contacts include DEP-1/CD148, PTP-μ, PTP-κ, SHP1, and SHP2.[132]

Another important protein regulating the integrity of endothelial cell–cell contacts is junctional eNOS.[95,133] Junctional eNOS is constitutively active and required for maintenance of junction integrity.[133,134] Both VEGFR-2 and PKA-c signaling have been implicated in the regulation of junctional eNOS and VE-cadherin stability.[93,135] In response to VEGF, junctional eNOS and VEGFR-2 disappear from lateral membranes, and there is a concomitant rearrangement of the actin cytoskeleton that increases cell permeability. In subconfluent cells, NOSTRIN is exclusively intracellular, but in confluent vascular endothelia, it is present at the lateral membranes, suggesting that it may also regulate junctional eNOS.[98] Given that endothelial cell junctions undergo remodeling in response to shear stress, it will be of interest to determine how NOSTRIN and eNOS localization and function are affected by mechanical stimulation or hypertension. Junctional eNOS is not associated with caveolin-1, yet overexpression of caveolon-1 in mice impairs VEGF-stimulated vascular permeability and angiogenesis; therefore, it remains an open question whether eNOS activation at cellular junctions versus in caveolae is governed by distinct mechanisms.[45,135] This is an important issue in understanding how the regulation of cell–cell adhesion and eNOS signaling is associated with mechanostimulation and inflammation.

RTK Trafficking as Therapeutic Target

As highlighted in previous sections, targeting of activated RTKs for degradation is a critical step in regulating the degree and duration of growth factor–induced signaling. Therefore, activation of endocytic trafficking pathways that selectively lead to inactivation and/or degradation of RTKs is an attractive therapeutic strategy. For instance, the predominantly neutralizing VEGF receptor VEGFR-1 has a 10-fold higher VEGF-binding affinity than that of VEGFR-2, but its expression in endothelial cells is very low. Therefore, upregulating VEGFR-1 surface expression could target the majority of VEGF for degradation and significantly reduce its mitogenic effect. Activation of downstream trafficking proteins that favor degradation of ligand-bound RTK is another therapeutic option to downregulate RTK-mediated signaling. Although the involvement of c-Cbl in targeting several RTKs for degradation may limit its use as a specific tool, it can be used as a more general inhibitor of RTKs. In contrast, the effect of Nedd4 on RTK signaling and trafficking varies from 1 receptor to the other and, in some cases, like EGFR and VEGFR or EGFR and IGF-1R, is quite the opposite. This makes Nedd4 an attractive target for receptor-specific modulation of signaling and trafficking pathways.[72,73]

Positive regulators of RTK activity that can be used to augment the duration and intensity of receptors signaling include Sprouty-1 and 2, Alix, LRIG-1, Grb10, and LRP1. Grb10 in particular can enhance (IGF-1R) or inhibit (VEGFR-2 and EGFR) degradation, depending on the type of RTK, and, like Nedd4, can be used for receptor-specific regulation.[65,71] Modulation of specific Rab protein functions to enhance or diminish endocytic trafficking along specific routes is currently achieved by overexpression or small interfering RNA (siRNA) treatment and has been reviewed by our group recently.[136] Efforts to develop small molecule inhibitors of specific E3 ubiquitin ligases is under active development.[137] With many new small molecule screening centers coming online through the NIH Roadmap, it is likely that new chemical modulators that either inactivate or activate specific trafficking machinery components will be identified that may serve as new therapeutics.

Continuous stimulation of endothelial cell with VEGF leads to depletion of VEGFR and makes the cells insensitive to further stimulation until newly synthesized receptors arrive at the surface.[138] This indicates that receptor recycling is not a major route for VEGFR recovery in endothelial cells. In some situations, such as to enhance wound healing or in diseases that require neovascularization (eg, ischemic heart disease), continuous activation of the VEGF/VEGFR pathway may be required. Therefore, strategies to stimulate recycling of internalized VEGFR2 by activating regulators of the recycling machinery, such as Rab4 and Rab11, could be an important therapeutic strategy. In ischemic heart disease, the leading cause of morbidity and mortality, the benefit of identifying such mechanisms to locally activate neovascularization alone or in harmony with surgical procedures, such as revascularization via coronary bypass graft surgery, will be an important advance. PlGF and some isotypes of VEGF specifically bind to only VEGFR-1. Therefore, continuous stimulation of endothelial cells with these isotypes before activation of VEGFR2 could specifically deplete VEGFR-1 from cell surfaces and help to augment signaling via VEGFR2.

In conclusion, RTK trafficking plays an enormous role in regulating the degree and duration of the various types of signaling that they mediate. Modulation of the trafficking process to either prolong or shorten signaling from the activated receptor provides novel therapeutic approaches for various diseases. To benefit from this huge potential, intensive investigation to elucidate the trafficking pathways and regulatory machinery governing RTK signaling and endocytosis is urgently needed.

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