Receptor Tyrosine Kinase endocytosis in endothelium: biology and signaling

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Abstract

Receptor tyrosine kinases (RTKs) are involved in regulation of key process in roles in endothelial biology including proliferation, migration and angiogenesis. It is now generally accepted that RTK signaling occurs intracellularly as well as on the plasma membrane although many important details remain to be worked out. Endocytosis and subsequent intracellular trafficking spatiotemporally regulate RTK signaling while "signaling endosomes" provide a platform for the compartmentalization of signaling events. This review summarizes recent advances in our understanding of endothelial RTK endocytosis and signaling using vascular endothelial growth factor receptor-2 as a paradigm.

Keywords

endocytosis; endosomes; signaling; VEGFR2; NRP1; synectin; Myosin-VI; FGFR1; EGFR

Introduction

Vascular networks reach every organ system in the body. In addition to supplying oxygen and nutrients to tissue as well as a variety of hormones, the vasculature is able to directly affect function of different organs via direct paracrine secretion of various molecules. Endothelial cells are the primary source of these factors and play a central role in most, if not all, kinds of biological activities, physiological or pathological, that take place in blood vessels. In addition, the endothelium plays a central role in the maintenance of normal vasculature, including regulation of its permeability, stability and proliferation.[1–3]

Endothelial cells respond to a variety of biochemical and mechanical inputs via a number of different receptor systems. Among these, receptor tyrosine kinases (RTKs) such as vascular endothelial growth factor receptor-2 (VEGFR2) and fibroblast growth factor receptor-1 (FGFR1) are essential regulators of such essential endothelial cell activities as proliferation,

Disclosures
None

migration, angiogenesis and vascular permeability. Whilst traditionally RTKs are thought to signal from the plasma membrane, recent studies have demonstrated that a number of critical signaling events occur in the cytoplasm, following RTK endocytosis, which bring them into close proximity with either downstream signaling targets or phosphatases.[4] Interestingly, the extent to which a specific RTK's signaling is affected by endocytosis is different for different receptors. This could be due to different endocytosis pathways or intracellular trafficking routes that these receptors take (Fig. 1).

To date, EGFR (epidermal growth factor receptor) has been one of the best-studied receptors in regard to the connection between its trafficking and signaling.[5] In addition to plasma membrane signaling, much of EGFR signaling output occurs from various endocytic compartments as its uptake and intracellular trafficking brings EGFR into association with a series of adaptor proteins involved in various signaling cascade.[6] In particular, EGFR activates ERK1/2 (extracellular signal-regulated kinases 1 and 2)[7] and AKT[8] signaling from EEA1-positive early endosomes.

Endocytosis of EGFR as well as of other receptors is regulated by the Rab family proteins.[9] The Rabs are GTP-binding proteins that switch between active GTP-bound and inactive GDP-bound forms. The switch between the two states is mediated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The Rab proteins regulate various stages of cellular transport, including vesicle budding, endosome trafficking, and tethering of vesicles with target membranes.[10] Different Rab proteins share structural similarity, but have unique subcellular localization and effector proteins, which enable them to be involved in different stages of cellular transport.[9]

Here we review the recent progress in the understanding of endocytosis and signaling of the two endothelial RTKs- VEGFR2 and FGFR1 and the effect this process has on their signaling and biological activities.

Endocytosis pathways of VEGFR2 and FGFR1

VEGFR2 is the primary signaling receptor for VEGF-A, the endothelial growth factor central to much of the blood vessel biology, as well as a related ligand VEGF-C. Following binding of its ligands, VEGFR2 undergoes dimerization and activation characterized by phosphorylation of key tyrosine residues in its cytoplasmic domain.[11] This is followed by endocytosis via the clathrin/dynamin-mediated pathway.[12] In addition to this ligand-dependent endocytosis, two other forms of VEGFR2 endocytosis have also been described: a slow constitutive recycling and shear stress-induced VEGFR2 endocytosis.

In unstimulated endothelial cells, VEGFR2 is located both on the plasma membrane and in EEA1-positive early endosomes where its presence is thought to be due to constitutive endocytosis.[13] The endosomal population of VEGFR2 undergoes short-loop Rab4-dependent recycling to the plasma membrane, rather than long-loop Rab11-dependent recycling.[14] The function of the constitutive VEGFR2 endocytosis and recycling remains unclear. Interestingly, ligand-independent VEGFR2 activation has been reported to occur in the Golgi of endothelial cells in response to hyperglycemia.[15] Further investigation of the

trafficking pathway involved could help our understanding of ligand-independent VEGFR2 functions.

Shear stress induces VEGFR2 endocytosis via phosphorylation of CD31 (PECAM), a transmembrane protein thought to be involved in shear stress sensing.[16] In this process, blood flow induces formation of a VE-cadherin–β-catenin–VEGFR2 complex[17] and aforementioned PECAM phosphorylation. Once phosphorylated, PECAM facilitates VEGFR2 transactivation by a Src family kinase[18] leading to activation of downstream signaling events such as ERK1/2 activation[19] and eNOS phosphorylation.[20] The shear stress-induced VEGFR2 endocytosis is caveolae-dependent[21] and the trafficking pathway involved is largely unknown.

Ligand (VEGF)-activated VEGFR2 endocytosis and subsequent intracellular trafficking is a tightly controlled process. The ligand-induced auto-phosphorylation of the receptor's kinase domain increases the rate of its internalization[22] that proceeds via clathrin-coated pits. AP2 and other adaptor proteins are involved in the recruitment of clathrin to plasma membrane, where clathrin undergoes polymerization, forming lattice-like coat.[23] Dynamin mediates the membrane fission and budding of clathrin-coated vesicles.[24] After internalization, the clathrin lattice is disassembled, leaving the uncoated vesicle to traffic towards endosomes.[23] VEGFR2 undergoes Rab5-dependent trafficking to early endosomes.[14, 25] In an unusual departure from conventional endosomal trafficking, VEGFR2 trafficking is in part dependent on the synectin-myosin-VI complex (Fig. 2A), as deletion of either molecule causes delayed VEGFR2 appearance in EEA1-positive early endosomes.[26]

Synectin (GIPC1) is a single domain PDZ protein that can bind to a number of transmembrane proteins and receptors including another VEGF-A receptor neuropilin-1 (NRP1), nerve growth factor receptor TrkA[13], non-tyrosine kinase fibroblast growth factors receptor Syndecan-4[27] and α5 and α6 integrins[28] among others. In addition to the PDZ domain, synectin also has the ability to bind intracellular motor protein myosin-VI (Fig. 2B). Myosin-VI is a unique member of the myosin family due to its ability to move towards the minus end of actin filaments. This allows it to move cargo from the plasma membrane into the cell along actin filaments. Myosin-VI plays a role in different stages of endocytosis, such as receptor internalization and sorting, through interaction with different binding partners.[29] Synectin, which associates with peripheral endocytic vesicles, binds myosin-VI and facilitates trafficking towards early endosomes. [30] The association between synectin and myosin-VI has been demonstrated in neurons[31] and epithelial cells[32–34], besides endothelial cells.[26, 35]

The recruitment of the synectin-myosin-VI complex to Rab5-positive endosomes usually involves adaptor proteins APPL1 and 2.[36, 37] However, in the case of VEGFR2, the role of APPL appears to be played by NRP1[25] that is able to bind VEGF-A_{165} via its extracellular VEGF binding site and myosin-VI via its cytoplasmic domain (Fig. 2B). In this model, VEGF-A is thought to form a complex with VEGFR2 and NRP1 that is then endocytosed and trafficked together. [38]

Once in early endosomes, VEGFR2 can undergo either lysosomal degradation or recycling back to plasma membrane. Approximately 40% of VEGFR2 is degraded within 30 minutes of VEGF stimulation[39] with the receptor sorted for lysosomal degradation via Rab7[39]. Proteasome- dependent degradation of VEGFR2 has also been reported that is, in part, regulated by VEGFR2-mediated activation of p38-MAPK that lessens the receptor's degradation by the 26S proteasome system.[40] The remainder of endocytosed receptors undergoes recycling. While constitutively endocytosed VEGFR2 may recycle in a Rab-4 dependent manner, VEGF stimulation switches VEGFR2 recycling to a Rab11-dependent pathway.[14]

NRP1 plays a critical role, not only in the recruitment of synectin in VEGFR2 endocytosis, but also in VEGFR2 recycling to the membrane. Stimulation of HUVECs with VEGF-A$_{165}$a that binds both VEGFR2 and NRP1 leads VEGFR2 to Rab11-dependent recycling, whereas stimulation with VEGF-A$_{165}$b which does not bind NRP1 causes Rab7-dependent degradation of VEGFR2 (Fig. 2C)[14]. In this manner NRP1 promotes both VEGFR2 endocytosis and recycling to the membrane and therefore relatively increases the proportion of VEGFR2 available for VEGF binding.

Similarly to VEGFR2, signaling by FGFR1, the principal FGF receptor in the endothelium, is also regulated by its endocytosis.[41, 42] The receptor's endocytosis can proceed via a number of different pathways. Depletion of E-Syt2, an endocytic adaptor protein that interacts with both activated FGFR1 and the clathrin adaptor complex AP-2, inhibits FGFR1 endocytosis and signaling in *Xenopus* embryo, suggesting clathrin-mediated pathway.[41] FGFR1 was also reported to interact with caveolin-1 and the knockdown of caveolin-1 decreases FGF2-induced activation of AKT and ERK1/2 in ovine fetoplacental artery endothelial (oFPAE) cells.[43] Finally, in endothelial cells FGFR1 endocytosis can proceed via macropinocytosis.[3] Thus, it is likely that for FGFR1 the predominant mode of uptake is cell-type specific.

Once internalized, FGFR1 is found in Rab5/EEA1-positive early endosomes [3, 44] from where the majority of the activated receptors are sorted for degradation via Lamp1-positive late endosomes while a relatively small proportion is recycled back to the plasma membrane for further stimulation [45].

Endocytosis-dependent regulation of VEGFR2 signaling

One unique aspect of VEGFR2 endocytosis is its regulation by a number of transmembrane and cytosolic interacting proteins, which play a role in either VEGFR2 internalization or degradation. A group of such proteins involved in VEGFR2 internalization are Dab2, ephrin-B2 and PAR-3. Dab2[46], a clathrin-associated sorting protein, and the cell polarity regulator PAR-3 interact with the transmembrane protein ephrin-B2 and VEGFR2. Disruption of this interaction by silencing of Dab2 or PAR-3 causes reduced VEGFR2 internalization and impaired VEGF-induced angiogenesis. After RTKs are internalized into early endosomes, a proportion of the receptors are modified by ubiquitin and then sorted for lysosomal degradation. CCM3[47] and myoferlin[48] respectively associate with VEGFR2 in endothelial cells and serve to enhance VEGFR2 stability by preventing receptor degradation.

Apart from internalization and degradation, VEGFR2 signaling is regulated by protein tyrosine phosphatases (PTPs) such as VE-PTP and PTP1b. VE-PTP is a transmembrane phosphatase that associates via its extracellular domain with VE-Cadherin at endothelial cell-cell junctions.[49] VEGF stimulation causes the activation and translocation of VEGFR2 to the junctions where the receptor is silenced by VE-PTP. VE-PTP also abolishes tyrosine phosphorylation of VE-Cadherin mediated by VEGFR2 and thus enhances EC junction integrity.[50] Silencing of VE-PTP boosts VEGFR2 signaling activity while also increasing permeability.[51]

Unlike VE-PTP, PTP1b affects VEGFR2 signaling further away from the plasma membrane. PTP1b is an ER-membrane bound phosphatase with its catalytic domain facing the cytosol.[52] The extensive ER network that reaches cell periphery enables PTP1b to come in close proximity with internalized VEGFR2 as it undergoes intracellular trafficking. Delayed trafficking of VEGFR2 to EEA1-positive endosomes observed in synectin or myosin-VI null endothelial cells or in endothelial cells expressing a truncated NRP1 with its cytoplasmic domain deleted results in reduced VEGF-dependent activation of ERK1/2 pathway. [25, 26] This can be corrected by suppression of PTP1b expression or activity, thus suggesting that the delay in VEGFR2 trafficking exposes the receptor to prolonged dephosphorylation by PTP1b.[53]

It is unclear in which subcellular compartment VEGFR2 is dephosphorylated by PTP1b. Studies of EGFR-PTP1b interactions showed that endosomes and ER form close membrane contacts which is the possible site of contact between the two molecules.[54] On the other hand, a study of insulin receptor trafficking found the interaction between IR and PTP1b in a peri-nuclear endosomal compartment.[55] Thus it is likely that PTP1b interacts with different RTKs in different subcellular compartment or compartment interfaces. Since RTK signaling is regulated both spatially and temporally, when and where they are deactivated by protein tyrosine phosphatases has a critical impact on the downstream signaling events. The driving force behind the formation of these compartment interfaces and the recruitment of PTP1b to the contact sites remains to be elucidated.

Another interesting aspect of PTP1b function is its potential to regulate RTK endocytosis. The ESCRT-0 complex, which is composed of the ubiquitin-binding proteins STAM 1 and 2 and their binding partner Hrs, binds to ubiquitin moieties on RTKs and facilitate the formation of multivesicular bodies which then fuse with lysosomes[56]. STAM2 is a PTP1b substrate and the latter's knockdown increases STAM2 phosphorylation[57] which in turn influences its function and localization. Therefore by affecting STAM2 phosphorylation, PTP1b potentially affects the degradation of activated RTKs. It is unclear whether PTP1b specifically affects VEGFR2 lysosomal degradation. VEGF stimulation resulted in increased phosphorylation of Hrs and the co-localization of VEGFR2 with the HRS/STAM complex[13], suggesting the involvement of this protein complex in VEGFR2 trafficking.

Biology of RTK endocytosis in endothelium

Increasing evidence from a number of studies showed the essential role of endocytosis in the optimal activation of RTKs and subsequent signaling events. [58, 59] Endocytosis brings the

receptors into close proximity of downstream targets or keeps them away from the dephosphorylation of protein tyrosine phosphatases. Generally RTKs have more than one downstream signaling pathway, yet the pathways are not necessarily activated at exactly the same time point, or for the same duration, or at the same subcellular localization. The relationship between RTK endocytosis and signaling requires better knowledge of the localization of their downstream targets. It is likely that each downstream signaling pathway has a unique "signaling compartment" along the RTK trafficking route. Such "signaling compartments" enable tight regulation of activation and inactivation of the signaling pathways. Knowledge on this aspect of receptor biology is still largely insufficient. Current understanding of signaling compartments regulating VEGFR2 signaling pathways is discussed below.

ERK1/2 activation and angiogenesis

VEGFR2 mediates endothelial cell proliferation, angiogenesis, arteriogenesis and controls vascular lumen size via the activation of ERK1/2 [26, 60, 61]. VEGF activates ERK1/2 through the classical Raf-MEK-ERK signaling cascade (Fig. 2D) [62]. FGF2 also induces ERK1/2 activation [63]. And FGF2-dependent ERK1/2 signaling plays a key role in the maintenance of VEGFR2 expression [1]. In both cases, ERK1/2 activation depends on receptor internalization, as inhibition of internalization abrogates VEGF- and FGF2-induced ERK1/2 signaling[64].

Knockdowns of Rab7 or Rab11 increase VEGF-induced ERK1/2 activation[26]. As these interventions, respectively, cause reduced degradation or recycling of VEGFR2, this suggests that ERK1/2 signaling occurs in a subcellular compartment preceding these compartments. ERK1/2 signaling has been associated with clathrin-coated vesicles (CCVs), early endosomes and late endosomes. Evidence for the role of CCVs and early endosomes comes from studies of neurotrophin receptor TrkA in neurons. A number of signaling components of the Ras-MAPK pathway, such as Ras, C-Raf, Mek and ERK1/2, were found to be present in CCVs in unstimulated cells and phospho-ERK1/2 was significantly enriched in CCVs after NGF treatment [65]. On the contrary, AKT and phospho-AKT were not detectable in CCVs before or after NGF stimulation[65]. Phospho-ERK1/2 presence has also been demonstrated in Rab5-positive early endosomes using confocal microscopy and increased phospho-ERK1/2 in Rab5-positive endosomes was observed after NGF treatment[66]. Furthermore, ERK1/2 activation was demonstrated to occur in EEA1-positive endosomes in endothelial cells, as delayed trafficking of activated VEGFR2 to this endosomal compartment caused impaired ERK1/2 activation.[25] PI3K and phospho-AKT were also present in early endosomes[67]. The detection of ERK1/2 signaling in CCVs is notable, considering the transient phase of these vesicles and rapid assembly and disassembly of clathrin coats in the vesicles, making CCVs a seemingly unsuitable compartment for signaling events to take place.

Late endosome involvement in ERK1/2 signaling was reported in studies of EGFR signaling. The MEK1 partner protein MP1 and the late endosomal adaptor protein p14 form a scaffold complex that recruits MAPK to late endosomes[68]. Upon EGF stimulation, activated ERK1/2 was found to co-localize with p14 and MP1 on late endosomes. RNAi-

mediated silencing of MP1 or p14 caused reduced EGF-induced ERK1/2 activation[69]. Taken together, ERK1/2 signaling is found to span a variety of subcellular compartments, from CCVs to late endosomes.

AKT activation and endothelial cell survival

VEGF promotes endothelial cell survival or anti-apoptotic signaling pathways via the anti-apoptotic kinase AKT/PKB (Fig. 2D). Several studies have suggested that AKT activation requires VEGFR2 internalization and endocytosis to a similar extent as ERK1/2 activation.[26, 70, 71] As discussed above, AKT activation was not detected in clathrin-coated vesicles in studies of neurotrophin receptor TrkA in neurons.[65] Interestingly, in the context of EGFR signaling, AKT, by immunostaining, has been shown to be closely associated with two Rab5 effectors APPL1 and APPL2[72], which are located on a subset of Rab5-positive early endosomes.[36] A knockdown of APPL1 in zebrafish led to a strongly diminished IGF-induced AKT phosphorylation, whereas IGF-induced ERK1/2 phosphorylation remained largely unchanged.[72] This is in agreement with the findings that ERK1/2 signals in endosomal compartments other than just early endosomes. Therefore APPL1 depletion has a minimal impact on IGF-induced ERK1/2 activation. On the contrary, IGF-induced AKT signaling is confined to Rab5/APPL-positive endosomes. APPL1 is also involved in regulating signaling of EGFR [36], TrkA [13], and adiponectin receptors [73]. It is unclear whether APPL1 is also regulates VEGFR2 endocytosis and signaling, since NRP1 is the molecule in endothelial cells that mediates the recruitment of the synectin-myosin-VI complex to activated VEGFR2. It remains to be elucidated whether VEGFR2-induced AKT activation depends on APPL1 and 2, and whether the signaling endosome for AKT is receptor-specific.

Src activation, endothelial cell migration and vascular permeability

Activated VEGFR2 recruits the signaling adaptor TSAd (T-cell-specific adaptor) which promotes Src activation (Fig. 2D). Src mediates Src-FAK (focal adhesion kinase) signaling activity which is involved in endothelial cell migration and vascular permeability [74]. The process of cell migration includes cell polarization, lamellipodia formation, and the assembly and disassembly of focal adhesions while vascular permeability is associated with the integrity of EC junctions. Both types of activities involve signaling events in specialized compartments of endothelial cells and the endosomal trafficking and signaling events in these contexts are different from what is discussed above. Inactive Src resides in the perinuclear region[75] which, according to other studies,[76] is likely to be Rab7-positive late endosomes. In contrast, active Src is not observed in the perinuclear region suggesting rapid translocation upon activation.[76] Once activated, Src moves to focal adhesions or cell-cell contact sites[39] through the Rab11-dependent recycling pathway[75] or the ESCRT (endosomal-sorting complexes required for transport) pathway[71] according to different studies. Src becomes more active as it progresses from the perinuclear region to the plasma membrane, demonstrating an "activation gradient" along the trafficking route.[75] This finding suggests that Src activation only begins after it exits late endosomes and enters the recycling endosome. Compared to ERK1/2 and AKT which are activated in early endosomes upon VEGF stimulation, Src is activated in a later signaling compartment, probably on the VEGFR2 recycling route.

Conclusions

There has been tremendous progress in recent years in studies of spatial and temporal aspects of RTK signaling. It is now clear that RTK endocytosis and signaling are closely related events. Endocytosis regulates signaling spatiotemporally as cytoplasmic uptake and trafficking bring activated RTKs into close proximity to downstream signaling molecules and signaling regulators such as protein tyrosine phosphatases. As each RTK has several downstream signaling targets, each signaling pathway has a unique "signaling endosome" or "signaling compartment". It is further likely that these differ for different RTKs. Such setting allows for spatial or temporal separation of individual signaling pathway and may to a considerable extent determine specificity of each RTK signaling.

In endothelial cells, current evidence suggests that VEGFR2 undergoes VEGF-induced endocytosis via clathrin-mediated pathway [77], whereas FGF2-induced FGFR1 endocytosis is clathrin-independent [3, 43]. Although ERK1/2 activation has been reported to occur in multiple endosomal compartments, the bulk of signaling activity is likely to originate from Rab5/EEA1-positive early endosomes in endothelial cells [25, 26]. It is likely that VEGFR2, FGFR1 and other receptors have different downstream signaling endosomes. Further studies are needed to fully unravel signaling mechanisms of endothelial receptor tyrosine kinases.

Acknowledgments

Abbreviations

EGFR	epidermal growth factor receptor
VEGFR2	vascular endothelial growth factor receptor-2
NRP1	Neuropilin 1
FGFR1	fibroblast growth factor receptor-1
ERK1/2	extracellular signal-regulated kinases 1 and 2

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Significance

This review focuses on the endocytosis of specific RTKs in endothelial cells and summarizes in particular the current knowledge regarding VEGFR2 endocytosis and signaling. The rapidly changing understanding of how this key endothelial RTK operates provides new insights into biology of key endothelium-dependent processes such as angiogenesis and arteriogenesis and offer the possibility of new therapeutic approaches to currently some of the most intractable cardiovascular illnesses.
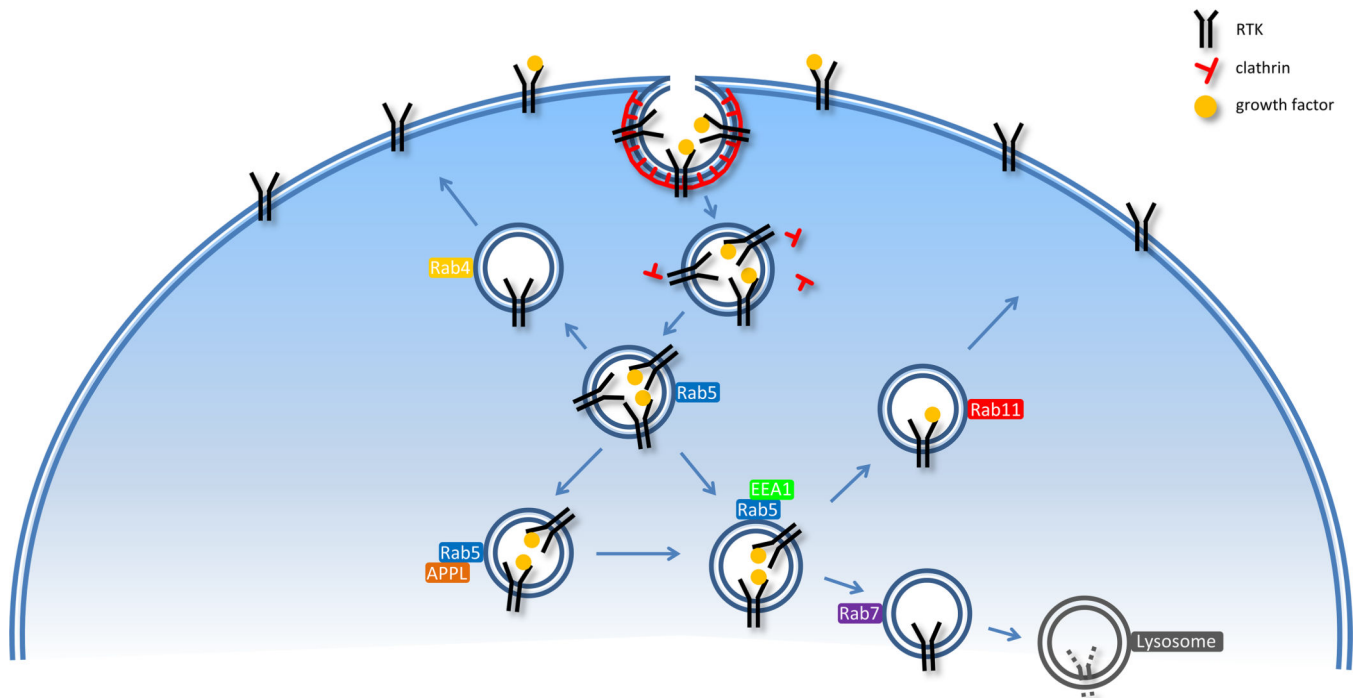
Figure 1.
General RTK endocytosis and recycling pathways. Ligand binding induces RTK activation and internalization (only clathrin-mediated pathway is shown). Internalized RTK undergoes trafficking to Rab5-positive endosomes, where they are sorted for either degradation or recycling. The degradation pathway is mediated by Rab7, whereas the recycling pathway is dependent upon Rab4 (short-loop) or Rab11 (long-loop). Specific details may vary for each particular RTK.
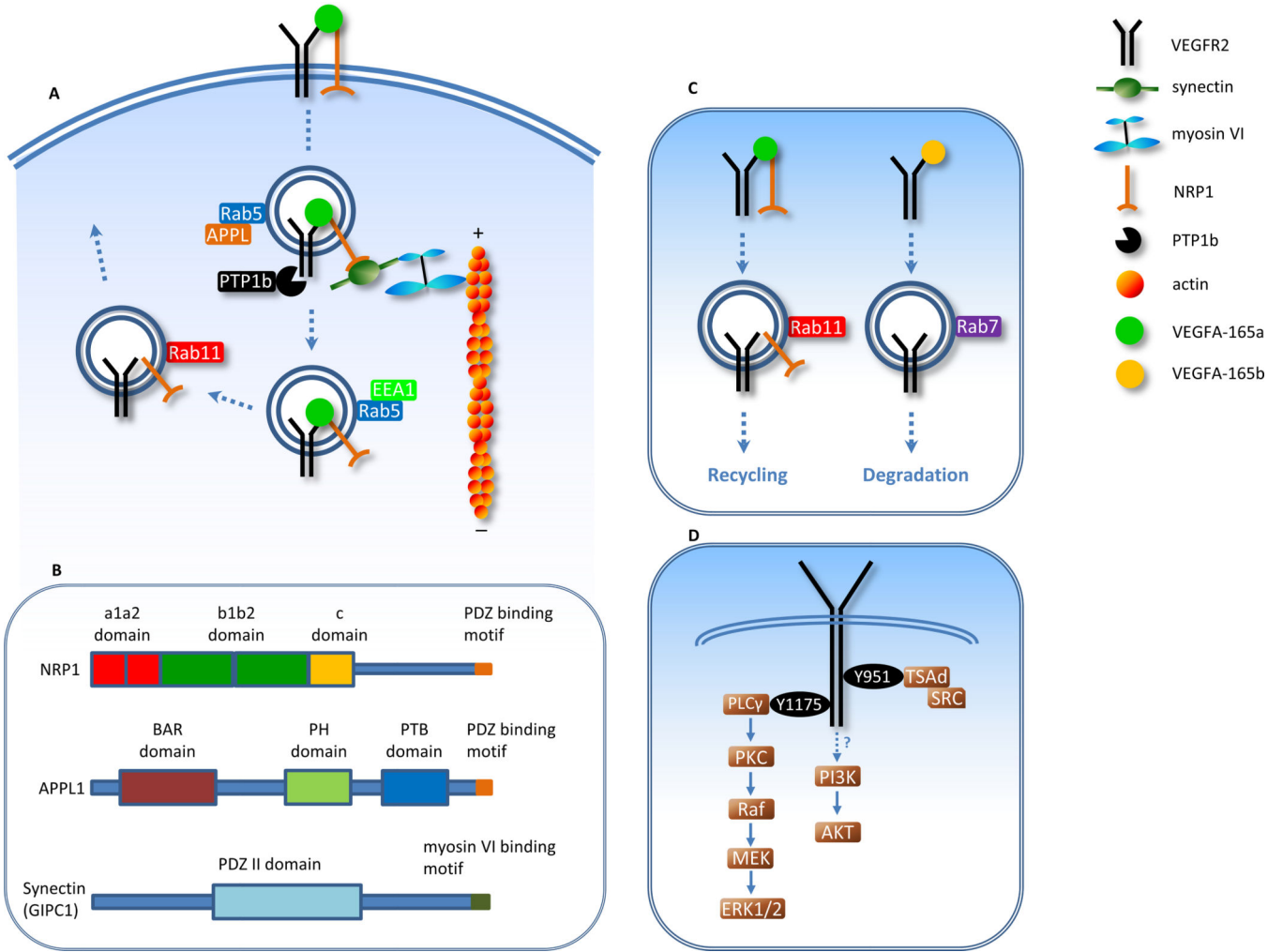
Figure 2.
VEGFR2 endocytosis and recycling pathways. (A) VEGFR2 trafficking requires the synectin-myosin-VI complex, which is recruited by NRP1 after receptor internalization. (B) The PDZ binding motif on NRP1 and APPL1 mediates binding to the PDZ domain of synectin. Synectin forms a complex with myosin-VI via its myosin-VI binding motif. (C) The internalized VEGFR2-NRP1 receptor complex undergoes Rab11-dependent recycling. In the absence of NRP1, VEGFR2 is degraded via Rab7-positive late endosome. (D) VEGFR2: Src activation is downstream of phosphorylation of tyrosine 951 and ERK1/2 activation is downstream of phosphorylation of tyrosine 1175. The mechanism of VEGF-induced AKT activation is unclear.