Constitutive Endocytosis of VEGFR2 Protects the Receptor against Shedding

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VEGFR2 plays a fundamental role in blood vessel formation and in life threatening diseases, such as cancer angiogenesis and cardiovascular disorders. Although inactive growth factor receptors are mainly localized at the plasma membrane, VEGFR2 undergoes constitutive endocytosis (in the absence of ligand) and recycling. Intriguingly, the significance of these futile transport cycles of VEGFR2 remains unclear. Here we found that, unexpectedly, the function of constitutive endocytosis of VEGFR2 is to protect the receptor against plasma membrane cleavage (shedding), thereby preserving the functional state of the receptor until the time of activation by VEGF. Inhibition of constitutive endocytosis of VEGFR2, by interference with the function of clathrin, dynamin, or Rab5, increases dramatically the cleavage/shedding of VEGFR2. Shedding of VEGFR2 produces an N-terminal soluble fragment (100 kDa, s100), which is released in the extracellular space, and a residual C-terminal part (130 kDa, p130) that remains integrated at the plasma membrane. The released soluble fragment (s100) co-immunoprecipitates with VEGF, in line with the topology of the VEGF-binding domain at the N terminus of VEGFR2. Increased shedding of VEGFR2 (via inhibition of constitutive endocytosis) results in reduced response to VEGF, consistently with the loss of the VEGF-binding domain from the membrane remnant of VEGFR2. These data suggest that constitutive internalization of VEGFR2 protects the receptor against shedding and provides evidence for an unprecedented mechanism via which endocytosis can regulate the fate and activity of growth factor receptors.

Results

Inhibition of Clathrin or Dynamin Blocks Efficiently the Constitutive Internalization of VEGFR2—At first, we confirmed (25) that as much as 40% of total cellular VEGFR2 is localized intracellularly (data not shown). Furthermore, to unambiguously study the importance of ligand-independent endocytosis of VEGFR2, we monitored internalization of the plasma membrane.
pools of VEGFR2, using an anti-VEGFR2 antibody uptake assay. Based on this approach, we found that the receptor internalizes efficiently in primary endothelial cells (HUVECs) under starvation.

2 The abbreviations used are: HUVEC, human umbilical vein endothelial cell; CME, clathrin-mediated endocytosis; CHC, clathrin heavy chain; TIRF-M, total internal reflection fluorescence microscopy; PNGase F, peptide N-glycosidase F; Endo H, endoglycosidase H; ER, endoplasmic reticulum; EEA1, early endosome antigen 1; ANOVA, analysis of variance.
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described endocytic pathway (2). Indeed, knockdown of clathrin heavy chain (CHC) resulted in a substantial decrease of VEGFR2 internalization (Fig. 1A). Because dynamin is a key regulator of CME (28, 29), we also tested whether inhibition of dynamin affects constitutive endocytosis of VEGFR2. To this end we used dynasore (30), a commonly used inhibitor of dynamin (31). Unlike knocking down or overexpression approaches, the use of rapidly acting inhibitors avoids the induction of compensatory internalization routes, which is a possible side effect in experiments where endocytosis is blocked by long-term treatment approaches (32). Treatment with dynasore caused a substantial inhibition of constitutive internalization of VEGFR2, as assessed by the antibody uptake assay (Fig. 1A), which is consistent with the data obtained by the knockdown of clathrin (Fig. 1A), or the overexpression approaches (shown in later experiments, Figs. 2 and 3). Additionally, we employed a surface biotinylation assay to assess biochemically the identity of the route of VEGFR2 internalization. Similarly to the immunofluorescence microscopy experiments, internalization of VEGFR2 was largely inhibited upon CHC knockdown (Fig. 1B), or by treatment with dynasore (Fig. 1C). On the other hand, knockdown of caveolin-1, which controls endocytosis via caveolae (33–37), plasma membrane invaginations that have been found to contain VEGFR2 (12, 38), had no effect on VEGFR2 endocytosis (Fig. 1B).

We also assessed constitutive internalization of VEGFR2 in live cells, using total internal reflection fluorescence microscopy (TIRF-M). In control cells, there was a quick disappearance of VEGFR2 positive patches from the plasma membrane, followed by appearance of new spots (supplemental Movie S1), a process that is reminiscent of vesicular internalization and recycling. In the presence of dynasore, there was a substantial inhibition of the vesicular movement of VEGFR2 (supplemental Movie S1). Combined, the above data suggest that VEGFR2 internalizes constitutively via the clathrin- and dynamin-dependent route.

Because the receptor internalizes constitutively (in the absence of VEGF), how are the surface levels of the receptor replenished to allow ligand binding and signal initiation? To study the efficiency of VEGFR2 recycling in endothelial cells, we employed a biochemical assay where the rate of receptor recycling is proportional to the rate of disappearance of an intracellular biotin-labeled VEGFR2 pool. We observed that the internalized receptor recycles effectively back to the plasma membrane (the amount of internalized VEGFR2 that undergoes recycling is ~5% per min, Fig. 1D). We also confirmed recycling of VEGFR2 using a microscopy-based approach that relies on receptor/antibody internalization, recycling, and re-internalization of the receptor (Fig. 1E, vehicle). When cells were treated with dynasore, the signal of VEGFR2 was largely inhibited (Fig. 1E, dynasore), suggesting that prior to recycling, the receptor internalizes dynamin dependently. Thus, these data suggest that, in the absence of VEGF, VEGFR2 undergoes continuous futile cycles of clathrin- and dynamin-mediated internalization and recycling.

Inhibition of Constitutive Endocytosis Results in the Formation of a Lower Molecular Weight Product (p130) of VEGFR2—
To get insights into the role of constitutive endocytosis of VEGFR2, we inhibited CME and tested the consequence on the fate of the receptor. Given that one of the main functions of endocytosis is to direct proteins to lysosomes or proteasomes for degradation (7–9), one would expect that inhibition of constitutive internalization of VEGFR2 would play a protective role on the fate of the receptor. Yet, surprisingly, inhibition of CME, either via lentiviral expression of a dominant-negative mutant of dynamin (K44A) (Fig. 2A) or via knockdown of CHC (Fig. 2B), resulted in a decrease of the levels of the mature form of VEGFR2 (230 kDa) and the production of a lower molecular mass (130 kDa) polypeptide. Knockdown of caveolin-1, which

![FIGURE 2. Inhibition of CME decreases the levels of full-length VEGFR2, whereas, concomitantly, it induces the generation of a lower molecular weight product (p130) of VEGFR2. A, HUVECs that were transduced with lentiviral vectors encoding dynamin 1 and 2 (dyn1/2 wt) or the K44A mutants of dynamin 1 and 2 (dyn1/2 K44A), or B, HUVECs that were transduced with siRNAs against clathrin heavy chain (CHC k.d.) or caveolin-1 (Cav1 k.d.), were lysed and subjected to immunoblotting analysis using a rabbit monoclonal anti-VEGFR2 cytoplasmic domain antibody. Western blots are representative of 3 independent experiments. C, HUVECs were treated with dynasore for the indicated time points and analyzed by Western blotting analysis using antibodies against the cytoplasmic domain of VEGFR2. The position of full-length VEGFR2, as well as the position of a lower molecular weight product of VEGFR2 (p130, shown by the arrow) and of EEA1 is indicated on the right of the blot. Densitometric analysis of p130 and full-length VEGFR2 is shown in the bar graph below the immunoblots (n = 4, mean ± S.D., * p < 0.05; ** p < 0.01; and *** p < 0.001, t test).](image)
had no effect on receptor internalization (see Fig. 1B), had no substantial effect on VEGFR2 levels (Fig. 2B).

To test whether the production of p130 is directly related to the decrease of the levels of full-length receptor, as well as to assess the kinetics of the production of p130, we determined the protein levels of VEGFR2 and p130 after acute inhibition of CME via dynasore, at increasing incubation times. The employment of a rapidly acting inhibitor (e.g. dynasore, which acts within about 20 min), rather than a knockdown or overexpression approach (which require 24 to 72 h of treatment), is critical for this type of experiment, because it allows almost immediate and direct assessment of the kinetics of the events altering the levels of VEGFR2 and p130, without the interference by new protein synthesis and/or protein degradation. Treatment of HUVECs with dynasore resulted in a time-dependent decrease of the levels of full-length VEGFR2 followed by a concomitant accumulation of p130 (Fig. 2C), which is in accordance with the results of K44A overexpression (Fig. 2A) or CHC knockdown (Fig. 2B) experiments. Importantly, the production of p130 was proportional to the decrease of the levels of full-length VEGFR2 (see diagram in Fig. 2C), indicating that the generation of p130 is directly linked to the decrease of the amount of mature VEGFR2. Notably, p130 was detectable, although at very low levels, even in the control/untreated cells (see the zero time point in the high exposure of the blot in Fig. 2C), whereas it became significantly induced upon inhibition of endocytosis (Fig. 2C). Together, the above data indicate that inhibition of constitutive endocytosis of VEGFR2 potentiates the generation of a lower molecular weight product of VEGFR2, the p130, at the expense of full-length VEGFR2.

Overexpression of Rab5S34N, Which Results in Higher Levels of VEGFR2 at the Plasma Membrane, Induces the Formation of p130—To further validate that the production of p130 is the result of the exposure of the receptor at the plasma membrane, we interfered with internalization of VEGFR2 via an independent approach, i.e. via modulation of the function of Rab5, which is a major regulator of the early endocytic pathway (5, 39 – 42) known to affect internalization of VEGFR2 (43). Here, we made use of two well studied mutants of Rab5, Rab5S34N and Rab5Q79L, which exert a differential effect on endocytosis (44, 45). Rab5S34N is a constitutively inactive, GDP-locked, mutant of Rab5 that inhibits endocytosis, whereas Rab5Q79L is a stably active GTP mutant that augments homotypic fusion and expansion of early endosomes, thereby causing retention of the endocytic cargo at the early endosome (44, 45). Overexpression of the dominant-negative mutant of Rab5 (Rab5S34N), by adenoviral infection, resulted in elevation of the levels of VEGFR2 at the plasma membrane and a parallel increase of the p130-kDa polypeptide, at the expense of full-length VEGFR2 (Fig. 3A). On the other hand, expression of Rab5Q79L, which caused accumulation of VEGFR2 at the early endosome, did not cause an increase of the levels of p130 (Fig. 3B). These data further indicate that the generation of p130 is due to the prolonged presence of the receptor at the plasma membrane.

Generation of p130 Is Not Due to Altered Processing in the Biosynthetic Route, or Due to Lysosomal/Proteasomal Cleavage—Subsequently, we sought to identify the mechanism that leads to the production of p130. VEGFR2 is a transmembrane glycoprotein with an aglycan size of 150 kDa. Glycosylation and further processing of VEGFR2 in the biosynthetic
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Compartments of the ER/Golgi network produces the mature form of 230 kDa (46). Given that interference with regulators of the endocytic routes may also affect transport within the biosynthetic pathway (47–50), which, consequently, may alter the glycosylation status of VEGFR2, we first tested whether the formation of p130 is due to altered glycosylation of VEGFR2 (e.g. due to lack of glycosylation or deficient carbohydrate processing). To this end, we tested the sensitivity of p130 to treatment with PNGase F, a glycosidase that cleaves N-glycosylation irrespectively of the maturation state of the glycans. Both p130 and full-length VEGFR2 were sensitive to PNGase F (Fig. 4A), excluding the possibility that p130 lacks glycosylation. We also tested the sensitivity of p130 to Endo H, a glycosidase that cleaves N-glycans of proteins that are within the ER or early Golgi compartments, but is unable to cleave mature carbohydrates of proteins that have passed beyond the Golgi network (51). p130 was resistant to treatment with Endo H (Fig. 4B), similarly to VEGFR2, suggesting that p130 is not an immature form of VEGFR2 in the ER/Golgi. The functionality of this glycosidase was confirmed by the observation that a 200-kDa minor fraction of VEGFR2 (running just below the mature form of VEGFR2, see Fig. 4B, vehicle), which contains immature glycans (46), is cleaved to a 150-kDa product (Fig. 4B, vehicle). All the above data suggest that p130 is not an incompletely processed pool of newly synthesized VEGFR2 in the ER/Golgi network, but is rather generated after the maturation and delivery of VEGFR2 to the plasma membrane.

We then tested whether p130 is a product of lysosomal or proteasomal cleavage of VEGFR2. Inhibition of lysosomal enzymes by chloroquine, or of the proteasome by lactacystin, did not reduce the levels of p130 (Fig. 4C), suggesting that the lysosomes or the proteasome are not responsible for the generation of p130. On the contrary, blockage of the lysosomal enzymes enhanced the levels of p130, indicating that, following its generation, p130 is degraded in the lysosomes (Fig. 4C). Consistently, upon longer treatment with the inhibitor of lysosomal enzymes, the levels of p130 became apparent even in the absence of any interference with endocytosis (Fig. 4D).

p130 Is Produced via Ectodomain Cleavage/Shedding of VEGFR2—Given that the production of p130 is augmented after prolonged exposure of VEGFR2 at the plasma membrane (Figs. 2 and 3), we reasoned that p130 might be produced via cleavage of VEGFR2 at the plasma membrane by matrix metalloproteases, a process called shedding. To test this possibility, we treated the cells with dynasore (to block endocytosis) in the absence or presence of TAPI-1, an inhibitor of matrix metalloproteases (52). Interestingly, TAPI-1 abolished the dynasore-mediated production of p130 (Fig. 5A). Consistently, in the presence of TAPI-1, the levels of full-length VEGFR2 remained stable even after 2 h of treatment with dynasore (Fig. 5A). These data suggest that p130 is a product of cleavage/shedding of VEGFR2 at the plasma membrane. Taking into account that p130 is detected using an antibody against the cytoplasmic domain of VEGFR2, and that it is glycosylated (Fig. 4A), we conclude that p130 is a transmembrane fragment of VEGFR2 that retains the cytoplasmic domain (which is detected by the antibody) and at least a part of the extracellular domain (which contains the sugars) (see scheme in Fig. 5B). These data indicate that p130 is produced via cleavage of VEGFR2 at the extracellular domain (Fig. 5B).
Given that, 1) the size of full-length VEGFR2 is 230 kDa, and 2) shedding of VEGFR2 produces a membrane-associated fragment of 130 kDa, ectodomain cleavage of the receptor should also produce a soluble fragment of 100 kDa (Fig. 5B), released in the tissue culture supernatants. To track the soluble fragment of VEGFR2, cell culture supernatants of dynasore-treated cells were collected, concentrated, and analyzed by Western blotting using antibodies against the extracellular domain of VEGFR2. Indeed, treatment with dynasore induced the release of a 100-kDa ectodomain fragment of VEGFR2 (s100) in the cell supernatants (Fig. 5C, left blot), which was abolished by TAPI-1 (Fig. 5C, left blot), consistently with the reasoning that VEGFR2 undergoes shedding at the cell surface. Importantly, the levels of s100 in the different samples of the cell supernatants (Fig. 5C, left blot) were analogous to the levels of p130 in the corresponding samples of the cell lysates (Fig. 5C, right blot), suggesting that s100 and p130 are produced by the same process (i.e., by shedding of VEGFR2). We also detected an additional, minor zone at 160 kDa (s160) (Fig. 5C, left blot), which based on its size, must correspond to a previously reported fragment (53).
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However, unlike s100, the levels of this minor fragment are not affected by dynasore treatment (Fig. 5C).

Binding of VEGF to VEGFR2 takes place at the N-terminal end of the ectodomain of the receptor. Thus, based on the apparent size of s100, the VEGF-binding domain of the receptor should be present in the soluble fragment (see Fig. 5B). To test this assessment, we employed a co-immunoprecipitation experiment between s100 and VEGF. To this end, tissue culture supernatants from HUVECs were supplemented with VEGF and incubated with anti-VEGFR2 antibodies that were raised against the extracellular domain of the receptor. Western blotting analysis of the pellet showed that the immunoprecipitated soluble fragment of the receptor (Fig. 5D, upper blot) pulls down VEGF (lower blot), suggesting that the released fragment of VEGFR2 contains the VEGF-binding domain of the receptor, further confirming that s100 is indeed a soluble fragment of VEGFR2.

Interestingly, s100 as well as p130 were also detected (despite their low levels) in untreated cells (see s100 and p130 in the vehicle samples of supernatants and lysates, respectively, in Fig. 5C). The presence of low levels of p130 in untreated cells was also observed in other experiments above (Fig. 2C, high exposure, zero time point; Fig. 3, A and B, control cells) (variability at the levels of p130 between different experiments is, at least in part, due to the different exposure times of the blots). Thus, although shedding is induced upon inhibition of endocytosis, it also takes place (yet, at low levels) even at steady state, i.e. in the absence of any cellular treatment. Because shedding of VEGFR2 occurs even without cellular treatments in HUVEC cultures, we questioned whether shedding occurs also in other types of endothelial cells, as well in intact vascular tissues. In agreement with the results in HUVECs, p130 was identified in bovine retinal endothelial cell cultures, as well as in umbilical cord extracts, in the absence of cellular treatments (Fig. 5E). Even though the levels of p130 were variable between the different types of endothelial cells, which could be due to differences in the rate of constitutive internalization of VEGFR2, or the rate of degradation of p130, or differences in the levels of matrix metalloproteases, identification of p130 in different cell types suggests that shedding of VEGFR2 takes place in different types of endothelial cells. Collectively, the above data suggest that VEGFR2 undergoes constitutive ectodomain shedding (Fig. 5, C–E), which produces a membrane-associated (p130) and a soluble fragment (s100), whereas inhibition of endocytosis increases substantially the levels of shedding (Figs. 2, A–C, 3, A and B, and 5, A–C). Enhancement of shedding by inhibition of receptor internalization suggests that endocytosis of VEGFR2 plays a protective role against cleavage/shedding.

Shedding of VEGFR2 Inhibits VEGF Signaling—Subsequently, we tested whether shedding influences VEGF-induced signaling to ERK1/2 and Akt. To this end, cells were treated with dynasore for 2 h, in the absence or the presence of the inhibitor of shedding (TAPI-1), washed extensively (to remove the drugs), and stimulated with VEGF (in the absence of the drugs) (Fig. 6). Consistently with the experiments shown above, dynasore-treated cells contained higher amounts of p130 and lower levels of full-length VEGFR2, in comparison to cells treated with dynasore in the presence of TAPI-1 (Fig. 6). As a consequence, VEGF-induced phosphorylation of ERK1/2 and Akt was significantly reduced in the cells experiencing higher shedding of VEGFR2 (Fig. 6). These data suggest that shedding is detrimental for VEGFR2 signaling, which is consistent with the finding that shedding removes the VEGF-binding domain of the receptor (shown above in Fig. 5, B–D).

Discussion

Although inactive growth factor receptors are, in general, mainly localized at the plasma membrane (1–3), VEGFR2 shuttles continuously between plasma membrane and endosomes.
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(Refs. 13, 17, and 25–27 and the present study). However, the role of these futile cycles has been unclear. Here we show that inhibition of constitutive internalization of VEGFR2, which prolongs the presence of the receptor at the plasma membrane, increases ectodomain cleavage of the receptor (shedding), by matrix metalloproteases. Thus, constitutive endocytosis of VEGFR2 plays a crucial role in providing the receptor with a route of escape from plasma membrane cleavage (shedding), thereby preserving the receptor in a functional state until the time of activation by VEGF. Importantly, shedding was detected, although at very low levels, even at steady state, in the absence of any interference (Fig. 2C, see high exposure of p130 blot, zero time point; Fig. 3, A and B, see p130 in control cells; Fig. 5C, see s100 in vehicle sample in supernatants and p130 in vehicle samples in lysates; Fig. 5E, see p130 in bovine retinal endothelial cells and umbilical cords), which must be due to the fact that a fraction of VEGFR2 resides at the plasma membrane (where metalloproteases are localized) at any given time. Differences at the levels of p130 between the above experiments are due to variations on the duration of the exposure of the blots and/or the amount of loaded samples. Interestingly, upon inhibition of endocytosis, which leads to a prolonged residence of the receptor at the plasma membrane, and, consequently, to a prolonged accessibility of the sheddases to the receptor, shedding is significantly enhanced. Shedding of the receptor produces a membrane-embedded product of 130 kDa and a released soluble fragment of 100 kDa, which contains the VEGF-binding domain of the receptor. As a consequence, when shedding is induced (e.g. upon inhibition of endocytosis), the response of the cells to VEGF is reduced. Thus, constitutive internalization of VEGFR2 is a protective mechanism against cleavage/shedding of the receptor. This protective role of endocytosis is surprising, because, one of the main functions of endocytosis is to direct molecules to lysosomes, or to the proteasome (7–9), for degradation, rather than to protect them from proteolytic cleavage.

Shedding of VEGFR2 may exert a bidirectional role in the regulation of endothelial cell signaling. Not only does it decrease the levels of functional receptor at the membrane, thereby limiting the ability of the cells to respond to VEGF, but also, it releases a soluble fragment (100 kDa), which retains its affinity for VEGF (Fig. 5, B–D). Consistently with our data showing that the 100-kDa released fragment sequesters free ligand, a smaller N-terminal fragment of VEGFR2 (75 kDa), produced by alternative splicing, was also able to bind and block VEGF (54). Thus, the soluble fragment of VEGFR2 could function as a ligand-trap (55) that limits the bioavailability of free VEGF. Collectively, the dual effect of shedding (decrease of the functional levels of VEGFR2 and blockage of free VEGF) could exert a cooperative inhibition on VEGF signaling.

There are striking differences between shedding of VEGFR2 reported here and the cleavage or shedding of VEGFR2 reported in previous studies. At first, cleavage at the cytoplasmic domain of VEGFR2 produced a cytoplasmic fragment (13, 15, 56), rather than the extracellular fragment that we found here. Two other studies reported shedding at the extracellular domain of VEGFR2, which appeared to play a positive role in signaling (57, 58). However, the shedding that we describe here inhibits downstream activation of ERK1/2 and Akt, consistently with the fact that the remaining part of the cleaved receptor (130 kDa) loses the VEGF-binding domain (Fig. 5, B–D).

Besides, in the report of Swendeman et al. (57), shedding produced a soluble fragment of 150 kDa, whereas the shedding process that we identified here produces a 100-kDa fragment (Fig. 5, B–D). Unfortunately, we cannot compare the size of the released fragment found here (100 kDa) with that of the study by Donners et al. (58), due to lack of the relevant information in the above report. In another study, a soluble 160-kDa fragment was found in the conditioned media of cultured endothelial cells (53), yet, it was not determined whether this was a product of shedding or a consequence of alternative splicing. Although we confirmed the presence of a soluble fragment of 160 kDa in the conditioned medium, this was a minor band in comparison to the major 100-kDa band that was produced upon inhibition of endocytosis (Fig. 5C). Besides, we did not observe differences at the levels of the 160-kDa band upon blockage of endocytosis. Finally, a recent study reported that deletion of cdc42 up-regulates VEGFR2 shedding, which resulted in the production of a 75-kDa membrane-associated fragment (59). In the Western blots of the cell lysates of this study (59) there was also a zone at 130 kDa, independently of the interference with cdc42. However, the source or the role of this 130-kDa zone was not studied (59), which does not allow us to conclude whether it represents the same product that we identified here. All in all, based on the characteristics of the shedding described here (role in signaling, dependence on endocytosis, and size of produced fragments), it is concluded that it represents a previously unrecognized event.

The role of endocytosis on protein shedding has been studied earlier. Depending on the particular plasma membrane protein, endocytosis may exert a positive or a negative role on cleavage/shedding. Thus, shedding of protocadherins and γ-secretase cleavage of Notch was found to require endocytosis (60, 61), whereas shedding of CX3CL1, APP, and pro-amphiregulin was augmented upon inhibition of endocytosis (62–65). Thus, the present study adds VEGFR2 in the very short list of a few proteins (CX3CL1, APP, and pro-amphiregulin) that, by being endocytosed, are protected against shedding. In fact, VEGFR2 emerges here as the first receptor, to our knowledge, whose shedding is influenced by endocytosis. Future studies will reveal whether other receptors share a similar mechanism of regulation.

Endocytosis is known to regulate receptor signaling via two independent mechanisms: 1) it delivers receptors, as well as other signaling molecules, to specific endosomal compartments, thereby controlling the assembly and activation of signaling complexes (2, 4, 5), and 2) it directs the ligands and/or receptors to lysosomes (8, 9), which causes irreversible termination of signaling. The present study, which focuses on the role of constitutive endocytosis of VEGFR2, adds a novel, third mechanism to the above list of means via which endocytosis can regulate receptor signaling: internalization of VEGFR2 allows the receptor to escape from ectodomain cleavage (shedding), thereby preserving the functional state of the receptor. Thus, endocytosis-dependent protection of VEGFR2 provides new insights in the overall role of endocytosis in the adjustment of endothelial cell signaling.
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Experimental Procedures

Reagents and Antibodies—The concentration of the reagents used in this study, unless stated otherwise, is shown below in parentheses. Dynasore (100 μmol/liter), lactacystin (10 μmol/liter), and chloroquine (100 μmol/liter) were from Sigma, whereas TAPI-1 (25 μmol/liter) was from Calbiochem. Mouse and goat anti-VEGFR2 extracellular domain monoclonal antibodies were from Abcam and R&D Systems, respectively, whereas the rabbit monoclonal and polyclonal antibodies against the cytoplasmic domain of VEGFR2 were from Cell Signaling and Santa Cruz Biotechnology, respectively. Recombinant human VEGF165 was obtained from Immunotools. The anti-actin antibody was from Millipore. Rabbit polyclonal antibodies against eEA1 and Rab5 were kindly provided by Marino Zerial (MPI-CBG, Dresden, Germany). Anti-clathrin heavy chain and anti-caveolin-1 antibodies were from BD Biosciences and Santa Cruz Biotechnology, respectively. The antibodies against ERK1/2, p-ERK1/2, Akt, and p-Akt were from Cell Signaling. Secondary antibodies coupled to Alexa fluorophores were from Invitrogen, whereas the HRP-conjugated antibodies were from Jackson ImmunoResearch. All other reagents were obtained from Sigma, unless stated otherwise.

Culture of Endothelial Cells, Transfections, Infections—HUVECs were isolated, cultured, and transfected or infected as described previously (66). Bovine retinal endothelial cells (obtained from VEC Technologies) were cultured on fibronectin-coated dishes, in DMEM supplemented with 10% fetal bovine serum, 4.5 g/liter of glucose, 1% penicillin, 1% streptomycin, and 1% heparin. siRNAs for human clathrin heavy chain (5'-GGGGUGCCAGAUUAUCAAUUtt-3') were from Ambion, whereas the siRNA for human caveolin-1 (5'-AAGAGCUUC-CUGAUUGAGAtt-3') and control siRNA (Random DS) were from Biospring. All knockdown experiments were carried out using 50 nmol/liter of siRNAs. Recombinant adenoviruses of Rab5Q79L and Rab5S34N were characterized previously (67). Lentiviruses of dynamin wt (1 and 2) or dynamin K44A (1 and 2) were generated according to a previously reported protocol (68). HUVECs were transduced at 50% confluence, in cell growth medium supplemented with 8 μg/ml of Polybrene and assayed 24–36 h post-transduction.

Treatments of the Cells with Inhibitors—Experiments were carried out using 2-h serum-deprived HUVECs, unless stated otherwise. HUVECs were treated with vehicle (dimethyl sulfoxide), dynasore, or dynasore + TAPI-1 for the indicated time points, lysed, and analyzed by Western blotting analysis using rabbit monoclonal anti-VEGFR2 cytoplasmic domain antibodies. To inhibit lysosomal or proteosomal degradation, prior to dynasore addition, HUVECs were pre-treated with chloroquine or lactacystin, respectively, for 2 h. To investigate the role of cell density on the regulation of VEGFR2 shedding, HUVECs were seeded at 5 × 10⁴ and 2 × 10⁵ cells/cm² to obtain confluent or sparse cultures, respectively. After 24 h, cells were treated with dynasore for 2 h and they were lysed and analyzed as described above. To investigate the role of VEGFR2 shedding on VEGF signaling, cells were treated for 2 h with dynasore, or dynasore + TAPI-1, washed 3 times with PBS, and the medium was replaced with serum-free M199 medium. After 10 min, cells were stimulated with 50 ng/ml of VEGF and they were lysed and analyzed by immunoblotting using anti-VEGFR2, anti-phosphorylated ERK1/2, and anti-phosphorylated Akt antibodies.

Indirect Immunofluorescence Microscopy and TIRF-M Live Cell Imaging—HUVECs were cultured in plastic dishes (Ibidi, 35-mm diameter) coated with collagen type I. Indirect immunofluorescence and analysis by confocal microscopy were employed as previously described (69). Images were captured using a Leica TCS SP5 II scanning confocal microscope and a Leica ×63 HXCL PL APO 1.4 NA objective. Data were subsequently processed in LAS AF according to the manufacturer’s guidelines. Live cell imaging of plasma membrane VEGFR2 was accomplished by TIRF-M. Cells were analyzed using a Leica AM TIRF MC set up on a Leica DMI6000 B microscope and a Leica ×100 HXCL PL APO 1.4 NA objective.

Internalization Assays—Following serum starvation for 2 h, HUVECs were transferred to 4 °C and the medium was replaced by ice-cold blocking buffer (1% BSA in serum-free M199 medium, buffered with 20 mmol/liter of Hepes). After a 30-min precooling step, cells were treated for 1 h with 10 μg/ml of mouse anti-VEGFR2 extracellular domain antibodies, at 4 °C. Cells were washed 3 times with ice-cold blocking buffer, treated for 30 min with vehicle or dynasore in blocking buffer at 4 °C, and transferred to 37 °C for a further incubation in the presence of 50 μg/ml of fluorescein isothiocyanate-conjugated transferrin (Invitrogen), for 15 min. Cells were acid-washed twice (ice-cold M199 medium, pH 2.0), washed 3 times with Ca²⁺/Mg²⁺-Hanks’ balanced salt solution, fixed, and processed for immunofluorescence microscopy. The above protocol was also applied to siRNA-treated cells.

For live cell TIRF-M analysis, cells were incubated with primary anti-VEGFR2 antibodies at 4 °C, followed by a 1-h treatment with Alexa Fluor 594-conjugated secondary antibodies, also at 4 °C. After three washes with ice-cold Ca²⁺/Mg²⁺-Hanks’ balanced salt solution, cells were treated for 30 min with vehicle or dynasore in a microscopy solution, at 4 °C, and transferred to a 37 °C chamber and analyzed by TIRF-M.

To assess biochemically the amount of surface or internalized VEGFR2, serum-starved HUVECs (2 h) were transferred to 4 °C and cell surface proteins were labeled with 0.5 mg/ml of cleavable EZ-Link Sulfo-NHS-S-S-Biotin (Thermo Scientific) in Ca²⁺/Mg²⁺-Hanks’ balanced salt solution for 20 min. Cells were washed 2 times with ice-cold 50 mM glycine in PBS, to quench unbound biotin, and treated for 30 min with vehicle or dynasore in serum-free M199 medium supplemented with 20 mmol/liter of Hepes at 4 °C. Then, the cells were incubated with pre-warmed media at 37 °C for 15 min, and were transferred back to 4 °C and incubated 2 × 15 min with biotin cleavage buffer (100 mM sodium 2-mercaptoethanesulfonate, 50 mM Tris, pH 8.5, 100 mM NaCl, 1 mM EDTA, 0.1% BSA), to strip biotin from the cell surface, non-internalized proteins. Sodium 2-mercaptoethanesulfonate was quenched with 50 mM iodoacetamide in PBS for 10 min and cells were lysed in lysis buffer (0.5% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA and protease inhibitors (complete mixture from Roche) and processed for pull-down using
streptavidin beads. Control samples were retained at 4 °C to analyze the surface levels of VEGFR2, as well as the efficiency of biotin cleavage. Prior to all incubations with buffers at 4 °C, cells were washed 3 times with ice-cold Ca²⁺/Mg²⁺-Hanks’ balanced salt solution. An identical protocol was applied to siRNA-treated cells.

Recycling Assays—To assess the rate of recycling of VEGFR2, we used an assay that has been previously described (70), with modifications. Briefly, cell surface proteins of HUVECs were labeled with cell-impermeable biotin at 4 °C, as described in the previous section, and the cells were transferred to 37 °C for 20 min to allow the internalization of biotinylated molecules from the plasma membrane. Subsequently, the cells were transferred back to 4 °C, treated with biotin cleavage buffer to strip biotin from non-internalized proteins, and the cells were transferred to 37 °C, for various time points, to chase the internalized biotinylated-VEGFR2 back to the plasma membrane. Finally, the cells were transferred to 4 °C, subjected to a second treatment with biotin-cleavage buffer, lysed, and processed for isolation of the proteins retaining biotinylation, using streptavidin beads. Under these experimental settings, the reduction of the intensity of the signal of biotinylated VEGFR2 (as a result of the second step of biotin cleavage) represents the amount of the receptor that has accomplished at least one round of internalization and recycling. To rule out the possibility that the reduction of the signal of biotinylated VEGFR2 is due to degradation of biotinylated receptors, control cellular samples were treated as above but were excluded from the second step of biotin cleavage.

Recycling of VEGFR2 was confirmed by confocal microscopy, as previously reported (26). Vehicle or dynasore-treated cells were incubated with mouse anti-VEGFR2 extracellular domain antibodies in serum-free M199 medium supplemented with 1% BSA, at 37 °C, in the presence of vehicle or dynasore. The receptor was allowed to internalize in complex with the primary antibody for 20 min and the cells were acid washed to strip plasma membrane-bound antibodies. Subsequently, the cells were incubated with secondary fluorescently labeled antimouse IgG antibodies for 20 min (during this time, recycling and re-internalization takes place), in the presence of vehicle or dynasore. At the end of the treatment, the cells were acid-washed again, fixed, and processed for immunofluorescence microscopy analysis. Under the above experimental conditions, the intracellular signal is detected only if the receptor accomplishes at least two rounds of internalization. At the first round, the antibody binds to VEGFR2 at the plasma membrane and internalization of the receptor protects the complex from the first acid wash. During the treatment of cells with the secondary antibodies, VEGFR2-primary antibody complexes recycle to the plasma membrane and bind to the secondary antibody. A second round of internalization protects VEGFR2-primary-secondary antibody complexes from the second acid wash, which results in the appearance of the fluorescent signal intracellularly.

**Endo H and PNGase F Treatment**—Total protein extracts (15 μg) of cells treated with vehicle or dynasore for 2 h were incubated with Endo H or PNGase F (New England Biolabs) for 12 h at 37 °C, according to the supplier’s protocol, and subjected to immunoblotting analysis using rabbit monoclonal anti-VEGFR2 cytoplasmic domain antibodies.

**Detection of Soluble VEGFR2**—Conditioned media of HUVECs treated with vehicle, dynasore, or dynasore + TAPI-1 for 2 h, in 100-mm diameter dishes, were collected and supplemented with 1 mmol/liter of EDTA, 2 mmol/liter of EGTA and protease inhibitors (complete mixture from Roche), and centrifuged at 6,000 × g, for 10 min, at 4 °C. The supernatants were concentrated by successive centrifugation in Ultracel YM-3 Centriplus and Centricon centrifugal filters (Millipore), according to the manufacturer’s instructions, and the concentrated samples were subjected to immunoblotting analysis using goat anti-VEGFR2 extracellular domain antibodies.

**Immunoprecipitation**—To investigate the interaction between VEGF and the soluble fragment of VEGFR2, conditioned media of 3-h serum-starved cells (8 × 100 mm-diameter dishes) were collected, combined, and concentrated to 0.6 ml, as described in the previous section. The sample was pre-cleared using protein G-agarose beads (Pharmacia Biotech) for 1 h at 4 °C and the supernatant was divided into two equal aliquots. The first aliquot was incubated with empty protein G-agarose beads, whereas the second aliquot was incubated with protein G-agarose beads bound to 10 μg of goat anti-VEGFR2 extracellular domain antibodies, for 4 h, to deplete soluble VEGFR2 (control sample). The samples were centrifuged (to remove the beads) and the remaining supernatants were incubated with 125 ng/ml of VEGF for 3 h at 4 °C. Then, the samples were incubated with 10 μg of goat anti-VEGFR2 extracellular domain antibodies, overnight at 4 °C, under rotation, followed by a further incubation with protein G-agarose beads for 4 h at 4 °C. The beads were pelleted, washed 3 times with PBS, and bound proteins were eluted by incubation with Laemmli buffer at 100 °C for 10 min. Proteins were separated by SDS-PAGE and analyzed by immunoblotting using goat anti-VEGFR2 and goat anti-VEGFR2 extracellular domain antibodies.

To investigate shedding of VEGFR2 in intact tissues, human umbilical cords from healthy donors were collected and the veins were cannulated with 20-ml blunt-tip syringes. The veins were perfused with excess of PBS to wash out blood, and incubated for 1 h with serum-free M199 medium at 37 °C. The medium was drained and the veins were incubated with 2 ml of ice-cold immunoprecipitation lysis buffer (50 mmol/liter of Tris, pH 7.4, 150 mmol/liters of NaCl, 1% Triton X-100, 1% Nonidet P-40, and protease inhibitors), by passing the solution several times through the vein. The solubilized extract was collected, centrifuged at 16,000 × g for 30 min, and the supernatants were pre-cleared with protein A-agarose beads, followed by incubation of the sample with 10 μg/ml of rabbit polyclonal anti-VEGFR2 cytoplasmic domain antibodies overnight at 4 °C under rotation. Immunocomplexes were precipitated using protein A-agarose beads and bound proteins were analyzed by Western blotting analysis using rabbit monoclonal anti-VEGFR2 antibodies.

**Quantification and Statistical Analysis**—Quantification of the immunoblots and the microscopy images was performed using ImageJ software. The values reported in the figures represent mean ± S.E. or S.D. calculated in at least 3 replicates for each experimental setting. Statistical differences were evalu-
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...continued using the Student’s t test, for two-group comparison, or by analysis of variance (ANOVA) followed by Bonferroni post hoc analysis for comparisons of more than two groups.

Author Contributions—D. B. and S. C. conceived and designed the study, analyzed and interpreted the data, and wrote the paper. D. B. performed all the experiments and prepared the corresponding figures.

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