Grb10 Prevents Nedd4-mediated Vascular Endothelial Growth Factor Receptor-2 Degradation

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One of the cellular mechanisms used to prevent continuous and enhanced activation in response to growth factors is the internalization and degradation of their receptors. Little is known about the molecular mechanisms involved in vascular endothelial growth factor receptor-2 (VEGF-R2) degradation. In a previous work, we have shown that the adaptor protein Grb10 is a positive regulator of the VEGF signaling pathway. Indeed, VEGF stimulates Grb10 expression, and Grb10 overexpression induces an increase in the amount and the tyrosine phosphorylation of VEGF-R2. In the present manuscript, we demonstrate that Grb10 stimulates VEGF-R2 expression by inhibiting the Nedd4-mediated VEGF-R2 degradation. First, we show that proteasome inhibition by MG132 induces an increase in VEGF-R2 amount, and that VEGF-R2 is ubiquitinated in response to VEGF. Phosphorylation of Nedd4, a VERT domain-containing ubiquitin ligase, induces the disappearance of VEGF-R2 in cells, suggesting that Nedd4 is involved in VEGF-R2 degradation. To determine whether Nedd4 directly ubiquitinates VEGF-R2, we expressed a ubiquitin ligase-deficient mutant Nedd4C854S. In the presence of Nedd4C854S, VEGF-R2 is expressed and ubiquitinated. These results suggest that VEGF-R2 is ubiquitinated but that Nedd4 is not involved in this process. Finally, we show that Grb10 constitutively associates with Nedd4. Co-expression of Nedd4 and Grb10 restores the expression of VEGF-R2, suggesting that Grb10 inhibits the Nedd4-mediated degradation of VEGF-R2. In this study, we show that Grb10 acts as a positive regulator in VEGF-R2 signaling and protects VEGF-R2 from degradation by interacting with Nedd4, a component of the endocytic machinery.

Vascular endothelial growth factor (VEGF) is a specific angiogenic factor. During embryonic development, VEGF plays an important role in vasculogenesis and angiogenesis. In adults, dysregulated angiogenesis is associated with pathological conditions, such as tumoral proliferation, rheumatoid arthritis, diabetic proliferative retinopathy, and inflammatory diseases. VEGF expression is mainly regulated by tissue oxygen content, but also by growth factors and cytokines, such as platelet-derived growth factor receptor (PDGF-R), insulin, IGF-I, tumor necrosis factor α, and transforming growth factor type β (1–5). VEGF stimulates vascular permeability, cellular migration and proliferation, and cell survival and angiogenesis through the activation of two tyrosine kinase receptors, VEGF-R1 or Flt1 and VEGF-R2 or KDR/Flik1. Although VEGF-R1 seems to be a non-signaling receptor, binding of VEGF to VEGF-R2 induces receptor dimerization, tyrosine kinase activation, and autophosphorylation of specific tyrosine residues. Activation of VEGF-R2 stimulates multiple signaling molecules, such as PI-3-kinase, MAP-kinase, PLCγ, FAK, Src, and STAT, leading to the generation of biological responses such as cell proliferation, migration, survival, and NO production (for review, see Ref. 6). We have shown that the adaptor molecule Grb10 is involved in a positive feedback loop in VEGF signaling. VEGF stimulates Grb10 mRNA expression in human umbilical vein endothelial cells (HUVEC), and in turn, ectopic expression of Grb10 induces an increase in the amount and the tyrosine phosphorylation of VEGF-R2 in HUVEC and in HEK-293 cells (7). Our hypothesis was that Grb10 inhibited VEGF-R2 degradation. However, the VEGF-R2 degradation pathway is not known. The aim of this work was to study the VEGF-R2 degradation pathway and to identify the role of Grb10 in this process.

Although it has been shown that VEGF stimulates VEGF-R2 degradation (8), the molecular mechanisms remain to be identified. However, VEGF-R2 is a receptor tyrosine kinase, and it is known that receptor tyrosine kinases are internalized by endocytosis through clathrin-coated vesicles, which fuse with an acceptor compartment to form early endosomes. Proteins are addressed to late endosomes or multi-vesicular bodies and are degraded in the lysosome by acid-dependent proteases (9). Ubiquitination of receptor tyrosine kinase is a signaling event leading the receptor to the degradative pathway. For instance, epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor recruit the E3-ubiquitin ligase cbl. cbl is tyrosine-phosphorylated and, in turn, induces ubiquitination of the receptors (10, 11). Receptors are mono-ubiquitinated, and this modification is sufficient for their endocytosis and degradation (12). Mono-ubiquitination of receptor tyrosine kinase

IR, insulin receptor; EGF-R, epidermal growth factor receptor; NT, N-terminal; IGF-I-R, insulin-like growth factor-I receptor.
targets it to lysosome degradation. In contrast, polyubiquitination targets proteins to degradation through the proteasome. Proteins involved in endocytosis are ubiquitinated. For example, in yeast, it has been shown that Vsp9, epsins, Eps15, and Hrs are ubiquitinated and are involved in endocytosis trafficking (13, 14).

Ubiquitination is a multi-step process that involves three enzymes. Ubiquitin is activated by E1 (ubiquitin activating enzyme) and is transferred to a ubiquitin-conjugating enzyme, E2, and then is linked to the substrate by an E3 ubiquitin ligase. There are two main E3 ubiquitin ligase families, the RING-domain and the HECT-domain-containing proteins (homologous to E6-AP C terminus) (15). E3s are domains involved in ubiquitin degradation. We show that ubiquitin from E2 to the substrate. Ned4 belongs to the HECT-domain-containing E3 protein, and ubiquitin is transferred from E2 to the HECT domain of E3, followed by transfer to the substrate. Ned4 is constituted of an N-terminal C2 domain responsible for Ca\(^{2+}\)-dependent binding of membrane phospholipids, multiple WW domains, which bind to proline-rich motifs, and a ubiquitin-protein ligase domain (16). Ned4 interacts with the epithelial sodium channel and down-regulates epithelial sodium channel activity in a ubiquitin-dependent manner (17). Ned4 has also been involved in the ubiquitination of endocytic proteins, such as Eps15 and Hrs (18, 19). Experiments done with yeast have shown that Rsp5 (the yeast homologue of the mammalian Ned4 family) is involved in the ubiquitination of endocytic proteins (20).

Grb10 is an adaptor molecule that contains several binding domains, such as a proline-rich region, a PH (pleckstrin homology) domain, an SH2 domain, and a BPS (between PH and SH2) region. Grb10 associates with numerous tyrosine kinase receptors such as epidermal growth factor (EGF) receptor, insulin, IGF-I receptors, VEGF-R2, c-kit, and with cytosolic proteins such as Raf1, MEK1, BCR-Abl, Jak2, Akt, and Ned4 (7, 21, 26). Grb10 is tyrosine phosphorylated by Tec, Src, and VEGF-R2 (7, 27, 28). Grb10 has controversial functions in growth factor signaling pathways. It has a positive action on mitogenesis in response to platelet-derived growth factor-BB (29). In contrast, Grb10 has been shown to have a negative action on insulin signaling. Its BPS domain associates with the catalytic domain of the insulin receptor (IR), and then prevents the tyrosine phosphorylation of substrates such as IR substrate-1 and IR substrate-2. This decrease in tyrosine phosphorylation is linked to a decrease in insulin-induced PI3-kinase and Akt activation (30). On the other hand, Grb10 associates with the p85 subunit of PI3-kinase, and expression of Grb10 induces an increase in insulin-induced metabolic responses (31). Finally, it has been shown recently that Grb10 stimulates IGF-I receptor ubiquitination by recruiting Ned4 to the receptor (32).

In the present work, we have studied the molecular mechanisms involved in VEGF-R2 degradation. We show that VEGF-R2 is ubiquitinated in response to VEGF and that its degradation is dependent upon the proteasome pathway. We demonstrate that Ned4 is involved in VEGF-R2 degradation. Finally, we provide evidence that Grb10 associates with Ned4 and that this association blocks the degradation of VEGF-R2.

EXPERIMENTAL PROCEDURES

Materials—VEGF was purchased from Peprotech (Tebu, France). We obtained antibodies to phosphothesine (clone 4G10) and VEGF-R2/KDR from Upstate Biotechnology (Mondolsheim, France); antibodies to Grb10 (sc-1026; sc-13955) from Santa Cruz Biotechnology (Tebu, France), antibodies to myc (clone 9E10) from Santa Cruz Biotechnology and Upstate Biotechnology; antibodies to HA (clone 12CA5) from Roche Diagnostics (Meylan, France) and HA.11 from Covance Research Products (Seraing, Belgium); antibody to Ned4 from BD Biosciences Pharmingen (Le Pont de Clais, France). Antibodies coupled to fluororescein isothiocyanate and Texas Red were obtained from Molecular Probes (BD Biosciences). Horseradish peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories (Montlucon, France). MG132 was purchased from Calbiochem (VWR International, Fontenay sous bois, France).

Oligonucleotides were purchased from Invitrogen (Cergy Pontoine, France) and enzymes were obtained from New England Biolabs (Beverly, MA) and Upstate Biotechnology. All chemical reagents were obtained from Sigma-Aldrich (Lyon, France).

DNA Plasmids—pcDNA3-Grb10-HA and pcDNA-VEGF-R2-myc have been described previously (7). Mouse Ned4 cDNA has been subcloned into KpnI-NotI sites in pcDNA4 vector (Invitrogen). Plasmids expressing HA-tagged proteins for insulin receptors have been described (32). pcDNA3-PDGF-R was obtained from C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) (34). Plasmid encoding for HA-tagged ubiquitin was obtained from Dirk Bohmann (University of Rochester, Rochester, NY).

Grb10-P3 mutant was generated by replacing proline residues 78, 81, and 83 by alanine, alanine, and serine, respectively. Grb10 NT mutant contains residues 1–353, Grb10 BS mutant contains residues 356–536, and residues 233–355 were deleted from Grb10 ΔPH. Ned4-C854S was generated by replacing cysteine residue 854 by serine. All mutants were generated by the QuikChange site-directed mutagenesis kit (Stratagene). Sequences of the constructs were checked by sequence analysis.

Culture—Human embryonic kidney cells (HEK-293 ENEA) were maintained in culture in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (Hyclone) and 500 μg/mL geneticin. HUVEC were isolated from umbilical cords by digestion with collagenase (35) or obtained from Clontech (Cambrex Bio-Sciences, Emeryville, CA). HUVEC were grown in EBM-2 supplemented with endothelial cell medium (Cambrex Bio-Sciences). HUVEC were tested positive for Von Willebrand factor immunostaining and were used before reaching passage 4.

Transient Transfection—Transfection of HEK-293 EBNA was performed by calcium phosphate precipitation (10 μg of DNA/9.5-cm\(^2\) dish). 16 h after transfection, the calcium phosphate-DNA precipitates were removed, and cells were incubated in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum. Before use, cells were serum-starved for 16 h in Dulbecco’s modified Eagle’s medium containing 0.2% (w/v) bovine serum albumin.

Immunoprecipitation and Western Blotting—Serum-starved cells were treated by ligands, chilled to 4 °C, washed with ice-cold phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 6 mM Na\(_2\)PO\(_4\), 1 mM KH\(_2\)PO\(_4\); pH 7.4), and solubilized with lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na\(_2\)PO\(_4\), 100 mM NaF, 2 mM vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, pH 7.4, 1% (v/v) Triton X-100) for 15 min at 4 °C. Cell homogenates were clarified by centrifugation at 15 000 ∗ g for 10 min at 4 °C. Cell lysates were incubated with antibodies and protein-G Sepharose. The pellets were washed twice with lysis buffer. Proteins were detected using SDS-PAGE and transferred by electroblotting to nitrocellulose membranes (Amersham Pharmacia Biotech, Orsay, France). The membranes were soaked first in blocking buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% (v/v) bovine serum albumin or 5% (w/v) nonfat milk and then in blocking buffer containing specific antibodies. After washes, proteins were detected using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Northern Blotting Analysis—Total RNA was isolated from 100-mm dishes using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Cergy Pontoine, France). RNA was denatured in formaldehyde and separated by electrophoresis in formaldehyde-containing agarose gel. RNA were transferred to Hybond-N membranes (Amersham Pharmacia Biotech). Probes were labeled with \(\gamma\)-32PdCTP by random priming using the Rediprime kit (Amerham Pharmacia Biotech) and purified with the Pheochromatin kit (Amerham Pharmacia Biotech). Hybridizations were performed at 42 °C in NorthernMax hybridization buffer (Ambion Inc., Cambridge, UK). Membranes were washed in 1× SSC, 0.5% (w/v) SDS and resolved on Storm 840 (Amerham Pharmacia Biotech).

Real-time Quantitative PCR—RNA was treated with DNase I and was reverse-transcribed using random priming and Superscript II according to the manufacturer’s instructions (Invitrogen). Quantitative PCR was performed by monitoring in real time the increase in fluorescence of SYBR Green on an ABI PRISM 7000 sequence detector system (Applied Biosystems) according to the manufacturer’s instructions. Gene-specific primers were designed using
and phosphotyrosine. Cell lysates were analyzed by immunoblotting using antibodies to myc.

4 h with MG132 (50 μM) prior to VEGF stimulation (5 min, 50 ng/ml). Cell lysates were analyzed by immunoblotting using antibodies to myc and phosphotyrosine.

RESULTS

The Proteasome Is Involved in the VEGF-R2 Degradation Pathway—We have shown previously that expression of Grb10 increased the expression and tyrosine phosphorylation of VEGF-R2 (7). To investigate whether Grb10 expression could increase translation of VEGF-R2, we inhibited protein synthesis by cycloheximide. HEK-293 cells were transfected with pcDNA3-VEGF-R2-myc and pcDNA3-Grb10-HA and treated with cycloheximide (100 μg/ml) for the indicated periods of time. B, HEK-293 cells were transfected with pcDNA3-VEGF-R2-myc with or without co-transfection of pcDNA3-Grb10-HA. Where indicated, cells were treated for 4 h with MG132 (50 μM) prior to VEGF stimulation (5 min, 50 ng/ml). Cell lysates were analyzed by immunoblotting using antibodies to myc and phosphotyrosine.

As the molecular mechanisms involved in VEGF-R2 degradation are not known, we investigated whether proteasomes could be implicated in this process. HEK-293 cells were transfected with VEGF-R2 and pretreated for 4 h with MG132, a proteasome inhibitor (Fig. 1B, left), or cells were transfected with VEGF-R2 and Grb10 (Fig. 1B, right). Cells were stimulated or not with VEGF, and lysates were analyzed by immunoblots with antibodies to myc to reveal VEGF-R2 and phosphotyrosine. We observed that VEGF stimulates tyrosine autophosphorylation of VEGF-R2. Long-term treatment with MG132 induced an increase in the amount and the tyrosine phosphorylation of VEGF-R2, even in absence of VEGF treatment. This result can be correlated with the observation that Grb10 expression increased VEGF-R2 expression and tyrosine phosphorylation. When cells are co-transfected with Grb10 and VEGF-R2 and treated with MG132, no additive effect was detected (data not shown). We conclude that the proteasome is involved in the VEGF-R2 degradation process.

VEGF Stimulates VEGF-R2 Ubiquitination—Ubiquitination of receptor tyrosine kinase is a molecular signal necessary for addressing the receptor to the degradative pathway. Thus, we have investigated whether in HEK-293 cells, VEGF-R2 is ubiquitinated in response to VEGF. HEK-293 cells were transfected with VEGF-R2 and ubiquitin-HA, treated for 5 min with VEGF, and cell lysates were subjected to immunoprecipitation with antibody to myc and analyzed by immunoblotting with antibodies to myc and HA.

Nedd4 Associates with Grb10—To identify the mechanism used by Grb10 to prevent VEGF-R2 degradation, we tested the binding of Grb10 to the E3 ubiquitin ligase, Nedd4. Indeed, a previous report has shown that mGrb10α associates with Nedd4 (21). HUVEC were treated with VEGF, and cell lysates were subjected to immunoprecipitation with a Grb10 antibody followed by Western blotting using Nedd4 antibody (Fig. 3A). We observed that in HUVEC, an association between Grb10 and Nedd4 is detected, which is not modulated by VEGF treatment.

To identify the functional domains of Grb10 involved in binding to Nedd4, we generated several mutants of Grb10. The SH2 domain of Grb10 has been mutated by replacement of the conserved arginine residue (R462K). The proline-rich region of Grb10 was mutated by replacing three proline residues (Pro78, Pro81, Pro83), and the following three deletion mutants were generated: (i) deletion of PH domain; (ii) expression of the N-terminal (NT) region (residues 1–353); and (iii) expression of the C-terminal region (residues 356–536) (Fig. 3B, see ΔPH, NT, and BS). These mutants were expressed in HEK-293 cells, and we tested their abilities to interact with Nedd4 by co-immunoprecipitation with an antibody to HA (Fig. 3, C and D). As observed in HUVEC, Grb10 associates with Nedd4. Mutation of Grb10 SH2 domain (R462K), proline rich region (P3), and deletion of the PH domain did not affect the ability of Grb10 to interact with Nedd4. In contrast, expression of the C-terminal part or the N-terminal region of Grb10 was not sufficient to allow the association between Grb10 and Nedd4. These results suggest that two domains of Grb10 are necessary for its association with Nedd4.

Nedd4 Is Involved in VEGF-R2 Degradation—Next, we in-
vestigated whether Nedd4 expression could regulate VEGF-R2 ubiquitination and degradation. Indeed, it has been shown that rsp5, the Nedd4 yeast homologue, is involved in the ubiquitination of endocytic proteins (20).

HEK-293 cells were transfected with VEGF-R2-myc and Nedd4 wild-type (WT) or a ubiquitin ligase-deficient mutant of Nedd4 (C854S). Cells were treated with VEGF, and lysates were analyzed by anti-myc immunoprecipitation followed by immunoblot with antibodies to myc or HA to detect the level of VEGF-R2 expression and ubiquitination (Fig. 4). We observed that VEGF stimulates ubiquitination of VEGF-R2. When cells were co-transfected with Nedd4 and VEGF-R2, VEGF-R2 was no longer detectable by anti-myc immunoblot. Expression of the catalytically inactive mutant of Nedd4 (C854S) restored the expression of VEGF-R2. Moreover, in the presence of the ubiquitin ligase-deficient mutant, Nedd4C854S, VEGF-R2 remained ubiquitinated. In conclusion, Nedd4 is involved in the cellular pathway leading to the degradation of VEGF-R2 but is not implicated in VEGF-R2 ubiquitination.

Next we examined the subcellular localization of VEGF-R2 and Nedd4 (Fig. 5). HEK-293 cells were transfected with VEGF-R2 and Nedd4 WT or mutant (C854S) and analyzed by confocal microscopy. In mock-transfected cells, immunolabeling for Nedd4 and VEGF-R2-myc showed no detectable signals (data not shown), indicating that Nedd4 is expressed at low levels in HEK-293 cells. In HEK-293 cells co-transfected with
Nedd4 and VEGF-R2, Nedd4 was mainly localized at the plasma membrane (Fig. 5, upper arrow). Labeling for myc shows that, as expected, VEGF-R2-myc was also expressed at the plasma membrane. However, in cells co-transfected with VEGF-R2 and Nedd4, we were not able to detect expression of either protein (see Fig. 5; Nedd4 is in green and VEGF-R2 is in red) in the same cell. This observation is in agreement with the fact that expression of Nedd4 inhibits VEGF-R2 expression, as shown in Fig. 4. Expression of the ubiquitin-ligase-deficient mutant, Nedd4C854S, allows us to detect both Nedd4 and VEGF-R2 in the same cell, which is consistent with the observation that Nedd4C854S did not stimulate VEGF-R2 degradation.

**Specificity of Nedd4 Action**—To determine the specificity of Nedd4 action, we tested whether expression of Nedd4 affects the expression level of other tyrosine kinase receptors, such as the IR and the PDGF-R (Fig. 6). HEK-293 cells were transfected with VEGF-R2, IR, or PDGF-R in the absence or presence of Nedd4. Lysates were analyzed with indicated antibodies. Our results show that Nedd4 is involved in the ubiquitination and degradation of proteins located at the plasma membrane or endocytic proteins; however, we cannot exclude that Nedd4 could regulate the stability of proteins implicated in transcription or stability of RNA. In the latter case, expression of Nedd4 could decrease the level of transcripts. Thus, we tested whether Nedd4 expression could affect receptor mRNA levels. HEK-293 cells were transfected with VEGF-R2, IR, or PDGF-R in the absence or presence of Nedd4. RNA were isolated and analyzed by Northern blotting using indicated probes (Fig. 7A). Expression of Nedd4 did not modify IR and PDGF-R mRNA levels. The transcript level for VEGF-R2 was detected by real-time reverse-transcription (RT)-PCR analysis (Fig. 7B); we found that Nedd4 expression did not significantly decrease the level of VEGF-R2 mRNA.

**Grb10 Expression Blocks Nedd4-induced VEGF-R2 Degradation**—To determine whether association between Grb10 and Nedd4 could affect Nedd4-mediated VEGF-R2 degradation,
HEK-293 cells were co-transfected with VEGF-R2 and WT Nedd4 or mutant Nedd4C854S, in the absence or the presence of Grb10. Cell lysates were analyzed by immunoblot with antibodies to myc to detect VEGF-R2, and with antibodies to phosphotyrosine, nedd4, and HA to detect Grb10 (Fig. 8). Expression of Grb10 induced an increase in the amount and the tyrosine phosphorylation of VEGF-R2, even in the absence of VEGF stimulation. Expression of Nedd4 decreases VEGF-R2 protein level, whereas the catalytically inactive mutant of Nedd4 (Nedd4C854S) does not modify VEGF-R2 protein level. Co-expression of Grb10 with wild-type Nedd4 restores the expression of the VEGF receptor (compare lanes 2, 4, and 6). Moreover, in the presence of Grb10 and Nedd4, VEGF-R2 is able to autophosphorylate on tyrosine even in the absence of ligand. The same pattern is observed in cells expressing VEGF-R2, Grb10, and Nedd4C854S. These results suggest that Grb10 inhibits Nedd4-mediated degradation of VEGF-R2.

Next, we investigated whether the association between Grb10 and Nedd4 is necessary to inhibit Nedd4-induced VEGF-R2 degradation. We used a mutant of Grb10 (Grb10 NT) that is unable to interact with Nedd4 (Fig. 3). HEK-293 cells were co-transfected with VEGF-R2 and Nedd4 in the absence or presence of WT Grb10 or NT mutant. Cell lysates were subjected to immunoprecipitation with antibody to myc followed by immunoblotting with antibody to HA to detect ubiquitination of VEGF-R2 (Fig. 9A). Total cell lysates were analyzed by immunoblotting with antibodies to myc to detect VEGF-R2 and with antibodies to Nedd4 and Grb10 (Fig. 9B). By anti-myc immunoblot, we observed that Grb10 expression increased the VEGF-R2 protein level (lane 4) compared with VEGF-R2 transfected cells (lane 2). In contrast, Grb10 NT mutant did not modify VEGF-R2 expression level (compare lane 6 to lane 2). Co-expression of Nedd4 and VEGF-R2 induced a decrease in VEGF-R2 protein level (lane 3 to lane 2). This decrease in VEGF-R2 protein level was inhibited when cells co-expressed VEGF-R2, Nedd4, and Grb10 (lane 5). In contrast, Grb10 NT mutant did not block Nedd4-mediated VEGF-R2 degradation (lane 7). Moreover, we observed that VEGF-R2 was ubiquitinated (Fig. 9A), and that its ubiquitination was directly proportional to the amount of receptor.

**DISCUSSION**

In the present study, we investigated the molecular mechanisms involved in VEGF-R2 degradation. We demonstrated that VEGF-R2 is ubiquitinated in response to VEGF, and that the HECT-domain E3 ubiquitin ligase Nedd4 is involved in VEGF-R2 degradation but not in its ubiquitination. Finally, we identified Grb10 as an inhibitor of the Nedd4-mediated degradation of VEGF-R2.

To study the VEGF-R2 degradation pathway, we investigated whether proteasome activity was implicated. We showed that the proteasome is involved in degradation of VEGF-R2,
cell lysates were subjected to immunoprecipitation with antibody to Grb10. 

were analyzed by Western blotting using antibodies to myc, Nedd4, or of pcDNA3-Grb10-HA. 

VEGF-R2 degradation. A produces a disappearance of VEGF-R2 expression in HEK-293 
nated by Nedd4 (18, 19). Unexpectedly, Nedd4 expression in-

such as Hrs and Eps15 (42). These proteins are also ubiquiti-

proteins containing UIM (ubiquitin interacting motif) domain, 

For instance, mono-ubiquitinated receptors could interact with 

tyrosine-phosphorylated receptor tyrosine kinase through its 

ligase, which is involved in the ubiquitination of several pro-

This result is in agreement with a recent report showing that 

cbl mediates Flk-1/KDR ubiquitination in bovine aortic endo-

This result is in contrast with a recent study 

cbl ubiquitates numerous proteins involved in endocytosis, but the function of these ubiqui-

remains unknown. Indeed, Nedd4 ubiquitates numerous pro-

because long-term treatment of cells with MG132 induced an increase in the amount and the tyrosine phosphorylation of VEGF-R2 even in the absence of VEGF treatment. This phos-

phorylation is probably due to the fact that increased receptor expression led to its spontaneous dimerization, allowing its transphosphorylation and its subsequent activation. The obser-

vation that MG132 increased the VEGF-R2 amount can be correlated with previous reports showing that proteasome inhibitors prevent degradation of tyrosine kinase receptors, such as EGF-R, nerve growth factor receptor, PDGF-R, and hepatocyte growth factor receptor (Met) (36–38). Proteasome activity is required for delivery of receptors to the late endosomal compartment. Indeed, inhibition of proteasome by lactacytin causes retention of EGF-R in multivesicular bodies at early stages of formation (39). However, it is now well established that receptor tyrosine kinases are not degraded by the protea-

some, but that proteins involved in receptor tyrosine kinase trafficking are ubiquitinated and degraded through the proteasome.

Because receptor tyrosine kinase is mono-ubiquitinated, we determined the ubiquitination pattern of VEGF-R2. We show that VEGF stimulates ubiquitination of its receptor. This ubiqui-

It has been shown that Nedd4C854S is unable to ubiqu-

to ubiquitin, restored expression of VEGF-R2 and its ubiquiti-

mutant of Nedd4 (Nedd4C854S), which was obtained after 

microscopy. We verified that this effect is not due to a defect in the transcription of VEGF-R2. The ubiquitin ligase-deficient mutant of Nedd4 (Nedd4C854S), which was obtained after mutation of the conserved cysteine residue involved in binding to ubiquitin, restored expression of VEGF-R2 and its ubiquiti-

ntation. It has been shown that Nedd4C854S is unable to ubiqui-

itination Hrs (19). These observations show that Nedd4 is im-

licated in VEGF-R2 degradation, but that Nedd4 is not involved in VEGF-R2 ubiquitation.

The role of Nedd4 in receptor tyrosine kinase degradation remains unknown. Indeed, Nedd4 ubiquitates numerous pro-

in endocytosis, but the function of these ubiqui-

tinations remains unclear. Moreover, Nedd4 targets cbl for 

ubiquitination and degradation (43). By this mechanism, 

Nedd4 protects EGF-R from cbl-mediated degradation. It is 

striking that for VEGF-R2, the mechanism is distinct. Indeed, 

we observe that Nedd4 expression did not affect PDGF-R ex-

pression level (Fig. 6), probably because Nedd4 inhibits cbl-

mediated ubiquitination and degradation of PDGF-R, as it has 

been shown for EGF-R. In contrast, Nedd4 expression de-

creases up to 80% the expression level of VEGF-R2, suggesting that VEGF-R2 is degraded by a Nedd4-dependent mechanism. Our results can be correlated with the function of the drosophila homologue of Nedd4. Nedd4 associates with Commissureless, a transmembrane protein, and induces its ubiqui-

itination. In turn, Commissureless down-regulates the ex-

pression of the Roundabout receptor (44).

Grb10 is a positive regulator of the VEGF signaling pathway. Indeed, VEGF stimulates Grb10 expression in endothelial cells (7, 45), and expression of Grb10 induces an increase in the amount and the tyrosine phosphorylation of VEGF-R2 (Fig. 1 and Ref. 7). Because proteasome inhibition leads to the same result, we hypothesize that Grb10 could interfere in the deg-

radation pathway of VEGF-R2. Indeed, we show that Grb10 inhibits Nedd4-mediated VEGF-R2 degradation. This action is due to the direct association between Grb10 and Nedd4, be-

cause a mutant of Grb10 (Grb10 NT) which is unable to asso-

ciate with Nedd4 does not prevent Nedd4-mediated VEGF-R2 

degradation. This result is in contrast with a recent study 

showing that Grb10 is involved in ubiquitination and degrada-

tion of insulin-like growth factor-I receptor (IGF-I-R). In re-

sponse to IGF-I, Grb10 associates with IGF-I-R and brings 

Nedd4 into the vicinity of IGF-I-R, leading to its ubiquitination (32). In contrast, we have shown here that (i) Nedd4 does not directly ubiquitinate VEGF-R2, and (ii) Grb10 prevents VEGF-R2 degradation. In summary, our results show that VEGF-R2 and IGF-I-R have distinct degradation pathways.

We have observed that Grb10 and Nedd4 constitutively asso-

icate and that this association does not require an intact 

Grb10 SH2 domain. Vecchione et al. (32) have shown that 

Grb10 SH2 domain constitutively associates with Nedd4 in a 

phosphotyrosine-independent manner. This discrepancy could 

be due to the fact that we are not studying the same isoform of 

Grb10. We use human Grb10γ instead of mouse Grb10α. Our 

results suggest that there might be two binding sites in Grb10, 

one in the N-terminal and one in the C-terminal region of 

Grb10. However, we cannot rule out that only one domain of 

Grb10 binds to Nedd4, and that the rest of the protein is 

required for its correct conformation allowing the proper asso-

ciation between Grb10 and Nedd4.

One of the outstanding questions is to identify the exact 

molecular mechanism by which Nedd4 is involved in VEGF-R2 

degradation. It is possible that ectopic expression of Nedd4 

induces the activation of one or multiple steps of endocytosis 

leading to the lysosome compartment. Nedd4 ubiquitates 

Eps15, which is involved in clathrin-mediated endocytosis.
Eps15 is tyrosine phosphorylated in response to EGF, and this tyrosine phosphorylation is required for EGFR internalization (46). However, the role of Eps15 ubiquitination in endocytosis and degradation of tyrosine kinase receptors has not been elucidated. We can speculate that ectopic expression of Nedd4 leads to an increase in Eps15 ubiquitination, which in turn will augment an increase in the degradation pathway of VEGF-R2. It has been shown that Nedd4 does not bind to Eps15 (18). It is possible that Grb10 acts as a link between the two molecules. Indeed, we and others (21) show that Nedd4 and Grb10 associate as (Fig. 3), and Grb10 possesses a consensus sequence for interaction with Eps15. Eps15 is composed of three Eps15 homology domains which bind to the NPF motif (47). Such a sequence is present on Grb10, P*P*P*P*FPFFEL, and could be involved in binding with Eps15. Therefore, ectopic expression of Grb10 could disrupt the Nedd4-Grb10-Eps15 complexes, inducing a mislocation of one or both proteins of this complex, and then suppressing molecular events involved in tyrosine kinase receptor degradation. Overexpression of Grb10 leads to disruption of the proper cellular localization of Nedd4 and then prevents it from being in the vicinity of its natural substrates. Indeed, it has been shown that overexpression of Grb10 leads to its mislocalization to the cytosol (48).

The function of Grb10 as an inhibitor of endocytosis of VEGF-R2 is reminiscent of the function described for Sprouty. Sprouty2 has been shown to enhance the Ras/mitogen-activated protein kinase signaling pathway in response to EGF by preventing EGF-R endocytosis. Sprouty2 sequesters cbl and prevents EGF-R endocytosis. Sprouty2 sequesters cbl and prevents ubiquitination and degradation of EGF-R (10).

In conclusion, we provide evidence that Grb10 is involved in a positive feed-back loop in the VEGF-R2 signaling pathway. Indeed, we and others (7, 45) have shown that VEGF stimulates Grb10 expression in endothelial cells. In turn, Grb10 interferes with molecular processes involved in VEGF-R2 degradation by interacting with at least one of the components of the degradation machinery, Nedd4. Considering this, it is tempting to propose that Grb10 could be one possible means to inhibit VEGF signaling. Indeed, inhibition of Grb10 expression would increase VEGF-R2 degradation, leading to a reduced VEGF action and, hence, controlled angiogenesis. Misregulation of this mechanism could result in pathological angiogenesis seen in several disease situations.

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