N-Glycosylation regulates ligand-dependent activation and signaling of vascular endothelial growth factor receptor 2 (VEGFR2)

The tumor microenvironment and proinflammatory signals significantly alter glycosylation of cell-surface proteins on endothelial cells. By altering the N-glycosylation machinery in the endoplasmic reticulum and Golgi, proinflammatory cytokines promote the modification of endothelial glycoproteins such as vascular endothelial growth factor receptor 2 (VEGFR2) with sialic acid–capped N-glycans. VEGFR2 is a highly N-glycosylated receptor tyrosine kinase involved in pro-angiogenic signaling in physiological and pathological contexts, including cancer. Here, using glycoside hydrolase and kinase assays and immunoprecipitation and MS-based analyses, we demonstrate that N-linked glycans at the Asn-247 site in VEGFR2 hinder VEGF ligand–mediated receptor activation and signaling in endothelial cells. We provide evidence that cell-surface–associated VEGFR2 displays sialylated N-glycans at Asn-247 and, in contrast, that the nearby sites Asn-145 and Asn-160 contain lower levels of sialylated N-glycans and higher levels of high–mannose N-glycans, respectively. Furthermore, we report that VEGFR2 Asn-247–linked glycans capped with sialic acid oppose ligand-mediated VEGFR2 activation, whereas the uncapped asialo–glycans favor activation of this receptor. We propose that N-glycosylation, specifically the capping of N-glycans at Asn-247 by sialic acid, tunes ligand-dependent activation and signaling of VEGFR2 in endothelial cells.

Within the tumor microenvironment, inflammatory stimuli alter expression of endothelial cell-surface carbohydrates (1–4). Immunomediated mechanisms that alter glycosylation and influence endothelial cell signaling are implicated in acquired resistance to anti-angiogenic therapies, highlighting the convergence of immunosuppressive and pro-angiogenic signaling in the tumor microenvironment (5). Pro-inflammatory interferon-γ and interleukin (IL)2–17 have been shown to increase the expression of α2,6-linked sialic acid–containing carbohydrate epitopes on the endothelial cell surface, whereas immunosuppressive cytokines IL-10 and transforming growth factor-β1 reduce α2,6-linked sialic acid–containing carbohydrate epitopes on N-linked glycans (4). In a separate study, tumor necrosis factor-α and IL-1β were shown to alter endothelial surface N-glycosylation, and this correlated with increased monocyte adhesion (1, 6). Other published reports suggest that abnormal endothelial cell glycosylation and expression of lectins, which bind glycan epitopes, may aid the development of resistance to anti-angiogenic cancer therapeutics (7, 8). Inflammatory cues, by triggering changes in endothelial surface carbohydrate structures, may alter angiogenic signaling by modifying the properties of endothelial glycoproteins that are key mediators of signaling and adhesion.

Among the VEGF receptor subfamily, VEGFR2 is one of the most important RTKs involved in pro-angiogenic signaling (9–11). Angiogenesis, the formation of new blood vessels from pre-existing vessels, occurs in both physiological and pathological contexts and is required for tumor growth and metastasis (12, 13). Stimulation of VEGFR2 by VEGF-family ligands promotes endothelial cell proliferation, migration, survival, and permeability and induces normally quiescent endothelial cells to proliferate and sprout (9, 11–19). VEGF–VEGFR2 interaction represents the most critical event in the activation of VEGFR2 and its subsequent signaling. Once activated at the cell surface, VEGFR2 undergoes dimerization, trans-phosphorylation, the docking of signaling proteins to specific phosphorylated tyrosine residues on its cytoplasmic domain, and subsequent...
internalization and degradation (11, 20). Dysregulation of VEGFR2 signaling contributes to the development of abnormal tumor blood vessels, which often have loose cellular junctions and endothelial fenestrae (21, 22). Abnormal blood vessels contribute to tumor angiogenesis, inflammatory cell infiltration, metastasis, resistance to chemotherapeutic agents in tumors of diverse origin, and may also limit T-cell infiltration, with implications for the field of immunotherapy (18, 23–32).

The extracellular domain of VEGFR2 contains seven immunoglobulin-like (Ig) repeats with 18 potential N-glycosylation sites. Extensive work performed in the laboratory of Dennis and co-workers (33–35) suggests that RTK N-glycan number and branching regulate cell proliferation and differentiation and that highly-branched N-glycans on RTKs present epitopes for lectins, promote the formation of lectin “rafts” on the cell surface, which oppose receptor endocytosis, and ultimately prolong receptor signaling (36–38). There is evidence that N-glycans impart structure-specific activity to modified proteins (39, 40). Analyses of epidermal growth factor receptors (EGFR) suggest that, independent of lectin-mediated effects, specific monosaccharides attached to N-linked glycans via specific linkages regulate receptor dimerization: both sialylation and outer-arm fucosylation of EGFR N-linked glycans at specific sites within the receptor lead to decreased receptor dimerization and phosphorylation, whereas core fucosylation has the opposite effect (36, 37, 41). Intriguingly, inhibition of N-linked glycosylation with tunicamycin reduces RTK signaling in tumor cells (42–46). Understanding how changes in glycosylation lead to dysregulation of VEGFR2-mediated signaling is essential to the development of therapeutic strategies that facilitate tumor vessel normalization, prevent metastasis, and expand the reach of immunotherapy.

We previously surveyed the site-specific N-glycan occupancy and glycoform heterogeneity displayed by VEGFR2 and identified multiple sites bearing complex, sialylated N-linked glycans (47). Immune-mediated changes have been shown to alter the expression of α2,6-linked sialic acid–containing carbohydrate epitopes on the endothelial cell surface, as noted above (4). Work performed in the laboratory of Rabinovich and co-workers (7) demonstrates that glycan-binding galectins activate VEGFR-2 in a ligand-independent manner. We sought to understand how changes in endothelial cell glycosylation influence ligand-dependent VEGFR-2 signaling, as such changes have been observed in other RTKs, including EGFR (37). We hypothesized that glycosylation regulates ligand-dependent VEGFR2 activation and signaling by tuning the VEGF ligand–mediated dimerization of the receptor and, therefore, undertook a determination of the consequences of altered glycosylation on VEGFR2 signaling. Here, we demonstrate that VEGFR2 glycosylation at site Asn-247 regulates ligand-dependent receptor activation and signaling. We provide evidence that cell-surface VEGFR2 displays complex N-glycans at Asn-247 and that N-glycans at Asn-247 with α2,6-linked sialic acid residues oppose receptor activation, whereas asialo–glycans favor VEGFR2 ligand-mediated activation and signaling.

Results

Enzymatic removal of VEGFR2 N-glycans enhances ligand-mediated receptor activation

To explore whether or not N-glycosylation of VEGFR2 impacts receptor activation and signaling, we treated VEGFR2/PAE cells with the endoglycosidase PNGase F to eliminate N-glycans, stimulated the cells with VEGF ligand (100 ng/ml) over a period of 30 min, and then assessed the activation of the receptor via Western blotting using an anti-phosphotyrosine 1054 (pTyr-1054) antibody to determine whether VEGFR2 N-glycosylation is necessary for receptor activation (Fig. 1A). Tyr-1054 is located within the kinase domain of the receptor and undergoes auto-phosphorylation in response to ligand stimulation (48). VEGFR2/PAE cells treated with PNGase F showed higher levels of phosphorylation at Tyr-1054 compared with untreated cells at 5 min ($p = 0.017$) and 10 min ($p = 4.1 \times 10^{-4}$) after stimulation with VEGF. Addition of heat-denatured PNGase F to cellular growth medium showed a much lower response, similar to the increase in activation in response to VEGF we observed for the control/untreated cells (data not shown). Receptor phosphorylation (activation) in the PNGase F–treated cells was approximately 2- and 6-fold higher, at the 5- and 10-min time points, respectively, compared with the control. By the 30-min time point, the levels of both pTyr-1054 – VEGFR2 and total VEGFR2 decreased, consistent with the established paradigm that the activated ligand–VEGFR2 complex undergoes clathrin-dependent endocytosis followed by degradation or recycling (9, 11). An identical set of experiments was performed at 4 °C to reduce clathrin-mediated endocytosis (data not shown). At early time points (0, 5, and 10 min), the results of this second set of experiments were identical to those from the experiment performed at 37 °C, while at 30 min pTyr-1054 – VEGFR2 was present at high levels at 4 °C but low in the experiment performed at 37 °C. Therefore, it appears that under physiological conditions (37 °C), the majority of phosphorylated (pTyr-1054) VEGFR2 is endocytosed and degraded after ligand stimulation in both the control and PNGase F–treated cells after 30 min, consistent with the literature. Based on our observation that removal of N-glycans favors ligand-mediated activation, we hypothesized that VEGFR2 N-glycans disfavor receptor dimerization and phosphorylation.

We reasoned that if our initial observations were due to altered VEGFR2 dimerization, as opposed to differences in ligand binding, an in vitro kinase assay (performed in the absence of ligand) should produce an outcome similar to experiments in live cells stimulated with VEGF. Therefore, we isolated VEGFR2 from VEGFR2/PAE cells, treated half of the isolated VEGFR2 with PNGase F, and performed an in vitro kinase assay with 0, 0.1, or 1 μM ATP (Fig. 1B). When normalized to total VEGFR2, receptor activation was higher after treatment with PNGase F at both concentrations of ATP (0.1 and 1 μM), suggesting that N-glycosylation plays a role in receptor dimerization and activation. In Fig. 1B, the change in the apparent molecular mass of VEGFR2 after treatment with PNGase F is evident. We consistently observed lower levels of total VEGFR2 after treatment with PNGase F in vitro, suggesting that efficient removal of N-glycans leads to the destabilization...
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Figure 1. Enzymatic removal of VEGFR2 increases dimerization and activation. A, serum-starved semi-confluent VEGFR2/PAE control and PNGase F–treated (37 °C, 4 h) cells were stimulated with VEGF (100 ng/ml) for 0, 5, 10, and 30 min at 37 °C. Following treatment, cells were lysed and analyzed via Western blotting with phosphotyrosine 1054, total VEGFR2, and α-tubulin. At 0 min, p = 0.46 (not significant); at 5 min, p = 0.017; at 10 min, p = 4.1 × 10−3; and at 30 min, p = 0.038. B, in vitro kinase assay of VEGFR2 immunoprecipitated from VEGFR2/PAE cells. VEGFR2 was incubated at 37 °C, and PNGase F (500 units) was added for 1 h, followed by addition of ATP (0, 0.1, or 1 μM) for 15 min, followed by denaturation and Western blotting. At the 0 μM ATP, p = 0.17 (not significant); at 0.1 μM ATP, p = 0.022; and at 1 μM ATP, p = 0.025. C, VEGFR2/HUVEC(Tert) control and PNGase F–treated (37 °C, 4 h) cells stimulated with VEGF (100 ng/ml) for 0, 5, 10, and 30 min at 4 °C. At 0 min, p = 0.16 (not significant); at 5 min, p = 7.0 × 10−3; at 10 min, p = 0.041; and at 30 min, p = 0.020. For all experiments, Western blotting quantification of was performed using ImageJ (n = 3). All experiments were performed in triplicate. Controls are represented by circles, and PNGase F–treated samples are represented by triangles. Individual data points and standard deviations are shown. p values were calculated using a two-tailed t test. Rounded rectangles mark time points where statistically significant (p < 0.05) differences between the control and PNGase F–treated samples were observed.

of the receptor. However, in experiments in which live cells were treated with VEGFR2 a much smaller shift was observed, suggesting that N-glycans were only partially removed. This is likely because VEGFR2 is in its folded native state and protected by the plasma membrane in live cells. This is in contrast to VEGFR2 in the in vitro kinase activity assay, where VEGFR2 is no longer protected by the plasma membrane and is in a non-native state due to the presence of DTT, and therefore most/all N-glycosylation sites are likely accessible to PNGase F.

Because there are critical differences in N-glycosylation in porcine and human cells, most notably the presence of N-glycolyl-neuraminic acid in pigs and its absence in human N-glycans, we next sought to reproduce these results in a human cell line. We generated immortalized HUVEC(Tert) cells with ectopic expression of VEGFR2 and stimulated serum-starved VEGFR2/HUVEC(Tert) cells with VEGF after treatment with PNGase F. We added VEGF and then incubated the cells, while maintaining the temperature at 4 °C to prevent receptor endocytosis and degradation. PNGase F–treated cells had much higher levels of pTyr-1054–VEGFR2 compared with cells without PNGase F treatment (Fig. 1C), and the differences were statistically significant at 5 min (p = 7.0 × 10−3), 10 min (p = 0.041), and 30 min (p = 0.020). Results were consistent with our previous observations regarding the effect of PNGase treatment prior to stimulation of the porcine cells.

Mutagenesis of VEGFR2 N-glycosylation site Asn-247 leads to higher levels of ligand-mediated phosphorylation within the kinase domain at tyrosine 1054

The extracellular region of VEGFR2 is composed of seven immunoglobulin (Ig)-like domains. Previous structure- and mutation-based strategies indicated that multiple Ig-like domains within the extracellular region of the receptor likely play significant roles in VEGFR2 dimerization and activation, particularly the Ig-like domains 2 and 3 (D2 and D3) that accommodate the ligand, and Ig-like domains 4 and 7 (D4 and D7) that may position the cytoplasmic region during dimerization. Based on our observation that enzymatic removal of VEGFR2 N-glycans resulted in higher levels of ligand-mediated phosphorylation, we created a panel of VEGFR2 N-glycosylation site mutants to determine whether one specific glycosylation site plays a regulatory role in modulating VEGFR2 activation. PAE cells with ectopic expression of VEGFR2 single glycosylation site mutants were stimulated with VEGF, and Western blotting analyses were performed to assess VEGFR2 activation (Fig. 2B). Compared with the WT receptor, when normalized to total VEGFR2, the
VEGFR2 N247Q mutant exhibited an increase in phosphorylation at Tyr-1054 after VEGF stimulation for 5 min (Fig. 2C, p = 5.6 × 10^{-3}). None of the other D2 and D3 glycosylation site mutants displayed statistically significant changes in ligand-mediated activation. Ligand stimulation of WT and N247Q VEGFR2 over a time course (0, 5, 10, and 30 min) resulted in higher levels of VEGFR2 activation in the N247Q mutant at all time points (Fig. 2D and E). Stimulated VEGFR2 N247Q/PAE cells showed 4.7-fold higher phosphorylation at Tyr-1054 compared with WT VEGFR2/PAE at 5 min (p = 0.044) and 2.6-fold higher phosphorylation after 10 min (p = 0.015). This pattern was also observed in HUVEC(Tert) cells with ectopic expression of either the WT receptor or the N247Q glycosylation site mutant; stimulated VEGFR2 N247Q/HUVEC(Tert) cells showed 2.6-fold higher Tyr-1054 phosphorylation after 5 min (p = 0.013) and 2.2-fold higher phosphorylation after 10 min (p = 0.017) (Fig. 2F). This result offers strong evidence that N-glycosylation at Asn-247 is involved in the regulation of ligand-mediated activation of VEGFR2.

Cell-surface VEGFR2 has sialylated and fucosylated N-linked glycans at site Asn-247

We focused on determining which N-glycoforms are present within the cell-surface population of VEGFR2, as only the surface population of VEGFR2 has the potential to bind the VEGF ligand. We labeled VEGFR2/PAE cells with a membrane-impermeable, cleavable (i.e. disulfide-linked), biotinylation reagent, isolated surface and nonsurface VEGFR2, subjected the VEGFR2 fractions to proteolysis, and subsequently analyzed glycopeptides from each of these populations using nUPLC-MS/MS. We determined that the nonsurface population of VEGFR2 has a lower apparent molecular mass, with respect to the surface population of VEGFR2, by separation of the proteins via SDS-PAGE and detection via Western blotting.
with an antibody against total VEGFR2 (Fig. 3A). The molecular mass difference is likely due to the distinctions in the glycoform distributions of the surface and nonsurface VEGFR2 populations.

To characterize the N-glycosylation of surface and nonsurface VEGFR2 populations, we applied nUPLC-MS/MS with the goal of detecting and profiling the N-glycosylation sites flanking the ligand-binding site. We were able to detect glycopeptides covering sites Asn-145, Asn-247, and Asn-320, using both data-dependent and targeted analyses. The HCD tandem mass spectrum of VEGFR2 glycopeptide $^{244}$LVLNCTAR$^{251}$ \begin{equation} \text{HexNAc~Hex} \quad m/z \quad 1406.0755 \end{equation} obtained via targeted analysis, is shown (Fig. 3B and Figs. S1 and S2). At site Asn-145, minimally processed high mannose N-linked glycans were dominant in the nonsurface population, whereas complex, fucosylated and sialylated N-glycans were observed in the surface population of VEGFR2 (Fig. 3C and Figs. S3–S5). At site Asn-160, we detected high-mannose N-linked glycans in both nonsurface and surface VEGFR2 (Fig. 3D and Figs. S6–S8). At site Asn-247, high-mannose N-linked glycans were detected almost exclusively in the nonsurface population, whereas complex sialylated glycans, with and without fucose, were detected in the surface population of VEGFR2 (Fig. 3E and Figs. S9–S18). We were unable to detect any peptides or glycopeptides covering site Asn-320, a potential N-glycosylation site within VEGFR2 D3 that is more distant from the VEGF ligand-binding pocket. Chiodelli et al. (8) have proposed that sialylated glycoforms of VEGFR2 modulate VEGFR2 signaling by altering N-Glycans regulate VEGFR2 activation and signaling.
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receptor interaction with the VEGF ligand; therefore, we were particularly interested to observe that surface VEGFR2 has high levels of sialylated glycoforms at site Asn-247 and fewer at site Asn-145.

Removal of sialic acid from VEGFR2 N-glycans increases ligand-induced receptor activation

To assess the importance of N-glycan sialylation on VEGFR2 activation, we treated VEGFR2/HUVEC(Tert) and VEGFR2 N247Q/HUVEC(Tert) cells with a broad-specificity neuraminidase that removes α2,3-, α2,6-, and α2,8-linked sialic acid residues from N-linked glycans and then stimulated the cells with VEGF. Treatment of liver cells with the broad-specificity neuraminidase followed by VEGF stimulation led to an increase in Tyr-1054 phosphorylation in treated VEGFR2/HUVEC(Tert) cells compared with untreated WT (Tert) cells (Fig. 4, A and B). As neuraminidase-treated cells with WT VEGFR2 responded to VEGF stimulation in a manner similar to cells expressing the VEGFR2 N247Q mutant, and both showed higher levels of activation compared with untreated cells expressing the WT receptor, we conclude that the increase in receptor activation observed for the N247Q mutant occurs because sialylated N-glycans are not available to interfere with ligand binding. Thus, although asialo- and fucosylated N-linked glycans at site Asn-247 appear to favor VEGFR2 activation, Asn-247 sialylated N-glycans appear to hinder VEGFR2 activation.

Sialic acid residues modify N-linked glycans on their nonreducing end(s) and can be linked to galactose via either α2,3- or α2,6-linkages or to other sialic acid residues via α2,8-linkages. We observed singly-sialylated N-glycans on VEGFR2 site Asn-247 (Fig. 3E), and we therefore reasoned that VEGFR2 site Asn-247 sialic acid residues must be either α2,3- or α2,6-linked. To study the linkage(s) of sialic acid residues on site Asn-247 glycans, we immunoprecipitated VEGFR2 from VEGFR2/HUVEC(Tert) cells, treated immunoprecipitates with either 2,3- or 2,6-linked sialic acid residues. Next, we sought to determine whether mutagenesis of the 2,6-linked sialic acid residues at site Asn-247 hinder VEGFR2 ligand-mediated activation. A, Western blotting of serum-starved semi-confluent VEGFR2/HUVEC(Tert) cells subjected to either control conditions or treated with a broad-specificity (α2,3; α2,6; α2,8) neuraminidase. Following neuraminidase treatment, cells were washed twice with cell culture medium to remove neuraminidase and then stimulated with VEGF (100 ng/ml). Analyses were repeated (n = 4). B, quantification of A, with pTyr-1054 vs VEGFR2/total VEGFR2. The experiment was performed four times (n = 4), and error bars shown represent 1 S.D. from the mean. Significant p values are shown.

For stimulated cells, a comparison of VEGFR2(WT) treated with neuraminidase versus VEGFR2 N247Q yields p = 0.88; VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.89; VEGFR2 N247Q versus VEGFR2 N247Q treated with neuraminidase yields p = 0.98 (not significant). For nonstimulated conditions, VEGFR2(WT) versus VEGFR2(N247Q) treated with neuraminidase yields p = 0.33; VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.17; and VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.12 (not significant).

Mutation of Asn-247 increases VEGFR2 signaling

Our results so far suggested that sialylated N-glycans at VEGFR2 site Asn-247 may hinder ligand-dependent receptor activation; removal of these N-glycans via either enzymatic methods or site-directed mutagenesis resulted in higher levels of receptor activation at Tyr-1054 within the kinase domain of the receptor.

Next, we sought to determine whether mutagenesis of the VEGFR2 Asn-247 glycosylation site led to changes in ligand-dependent intracellular signaling. Following phosphorylation at Tyr-1054 within the kinase domain, VEGFR2 can also become phosphorylated at Tyr-1175 and can subsequently serve as a docking site for SH2 domain-containing proteins, including PLC-γ1, leading to increased endothelial cell migration and proliferation (9, 11). Therefore, we stimulated VEGFR2/PAE cells with VEGF and assessed phosphorylation of VEGFR2 at Tyr-1175 and PLC-γ1 at Tyr-783 via Western blot analysis (Fig. 5A). We observed higher levels of phosphorylation at VEGFR2 Tyr-1175 (p = 3.0 × 10^{-5}) and PLC-γ1 (p = 0.017) in VEGFR2 N247Q/PAE cells compared with VEGFR2/PAE cells after 5

**Figure 4.** α2,6-Linked sialic acid residues at site Asn-247 hinder VEGFR2 ligand-mediated activation. A, Western blotting of serum-starved semi-confluent VEGFR2/HUVEC(Tert) cells subjected to either control conditions or treated with a broad-specificity (α2,3; α2,6; α2,8) neuraminidase. Following neuraminidase treatment, cells were washed twice with cell culture medium to remove neuraminidase and then stimulated with VEGF (100 ng/ml). Analyses were repeated (n = 4). B, quantification of A, with pTyr-1054 vs VEGFR2/total VEGFR2. The experiment was performed four times (n = 4), and error bars shown represent 1 S.D. from the mean. Significant p values are shown.

For stimulated cells, a comparison of VEGFR2(WT) treated with neuraminidase versus VEGFR2 N247Q yields p = 0.88; VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.89; VEGFR2 N247Q versus VEGFR2 N247Q treated with neuraminidase yields p = 0.98 (not significant). For nonstimulated conditions, VEGFR2(WT) versus VEGFR2(N247Q) treated with neuraminidase yields p = 0.33; VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.17; and VEGFR2(WT) versus VEGFR2 N247Q treated with neuraminidase yields p = 0.12 (not significant).
min (Fig. 5, B and C). We also observed higher levels of phospho-p44 MAPK in the VEGFR2/N247Q/PAE mutant compared with the WT at 5 and 10 min ($p = 3.8 \times 10^{-4}$ and $p = 0.014$, respectively), when normalized to total p44 MAPK (Fig. 5, A and D). Therefore, mutagenesis of the Asn-247 glycosylation site not only resulted in higher levels of VEGFR2 activation, but also led to higher levels of downstream signaling via activation of PLC$_1$ and p44 MAPK signaling.

VEGFR2 site Asn-247 N-glycans hinder receptor dimerization, activation, and degradation

We next sought to determine whether changes in VEGFR2 activation resulting from mutagenesis of the Asn-247 glycosylation site could be explained by changes in VEGFR2 dimerization. VEGFR2 and other RTKs can undergo spontaneous disulfide bond formation following ligand-induced dimerization that results in the covalent linkage of receptor monomers followed by internalization and degradation (51–53). We reasoned that if higher levels of VEGFR2 N247Q undergo dimerization in response to stimulation, as compared with WT VEGFR2, we should also observe a decrease in VEGFR2 N247Q levels at the cell surface, resulting from increased internalization and degradation of the receptor. We stimulated serum-starved cells with VEGF, subjected them to biotinylation with sulfo-NHS-biotin, VEGFR2 immunoprecipitation, and Western blot analysis of whole-cell lysates, and then immunoprecipitated VEGFR2 (Fig. 6A). We found that, after 60 min, VEGFR2 N247Q/PAE cells had approximately half (62%) the amount of receptor at the cell surface compared with VEGFR2/PAE cells (Fig. 6B). Next, we stimulated VEGFR2/PAE and VEGFR2 N247Q/PAE cells with VEGF, lysed the cells, and performed SDS-PAGE and Western blotting analyses under both nonreducing and reducing conditions. We detected higher levels of VEGFR2 dimer (at $400$ kDa) in VEGFR2 N247Q/PAE cells compared with VEGFR2/PAE cells after 10 min of VEGF stimulation at 37 °C (Fig. S21). In this experiment, we also observed a decrease in total VEGFR2 after ligand stimulation, in both the WT and mutant receptor, consistent with receptor degradation. We propose a model whereby sialylated VEGFR2 N-glycans hinder VEGF ligand-mediated dimerization, activa-
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Figure 6. Mutagenesis of VEGFR2 N-glycosylation site Asn-247 results in increased receptor activation, dimerization, and degradation. A, semi-confluent VEGFR2/PAE and VEGFR2 N247Q/PAE cells were serum-starved overnight, followed by stimulation with VEGF (100 ng/ml) for 0, 10, 30, or 60 min, cell-surface labeling with sulfo-NHS-biotin, cell lysis, immunoprecipitation of VEGFR2, SDS-PAGE of whole-cell lysates and immunoprecipitated VEGFR2, and transfer to PVDF membranes. Anti-VEGFR2 was used to detect total VEGFR2, and avidin-HRP was used for detection of surface-labeled VEGFR2 in immunoprecipitated fractions. Anti-α-tubulin was used as a loading control (in whole-cell lysate). B, quantification of surface VEGFR2 (detected by avidin-HRP)/total VEGFR2 (n = 3) (p < 0.05). Values were normalized to the 0-min time point. At 60 min, p = 0.024. At 10 min, p = 0.38, and at 30 min, p = 0.15 (not significant). C, model of Asn-247 glycan hindrance of VEGFR2 activation, dimerization, and degradation. All experiments were performed in triplicate (n = 3), and error bars shown represent the standard deviation of the mean. Rounded rectangles mark time points where statistically significant (p < 0.05) differences between the WT VEGFR2 (control) and N247Q mutant were observed.

Discussion

VEGFR2 is a highly N-glycosylated RTK that drives endothelial cell proliferation, migration, survival, and permeability. VEGFR2 is required for tumor angiogenesis. Dysregulation of VEGFR2 signaling contributes to the development of abnormal tumor blood vessels, which often have loose cellular junctions and endothelial fenestrae (21, 22). Abnormal blood vessels contribute to tumor angiogenesis, inflammatory cell infiltration, metastasis, and resistance to chemotherapeutic agents in tumors of diverse origin, and they may also limit T-cell infiltration, with implications for the field of immunotherapy (4, 18, 23–32). Endothelial cells display altered glycosylation, depending on where they reside in the vascular system, and exhibit changes in glycosylation in response to secreted factors and inflammation in the tumor microenvironment (1–3). Published reports suggest that abnormal endothelial cell glycosylation and expression of lectins, which bind glycan epitopes, may explain the development of resistance to some anti-angiogenic cancer therapeutics (7, 8). Protein N-glycosylation is a complex co- and post-translational process that occurs in the ER and Golgi, and leads to the production of heterogeneously-modified populations of membrane and secreted glycoproteins (54). Historically, carbohydrate modifications to proteins have often been regarded as decorations with little or no functional significance, but there is growing awareness that changes in glycosylation alter cellular signaling (35, 37, 55) and adhesion (56–59) and play critical roles during embryonic development and in diseases arising from dysregulation of cellular signaling (60, 61), including cancer. In our study, we have carefully examined the contribution of sialylated N-glycans on site Asn-247 to VEGFR2 receptor signaling. Our results indicate that VEGFR2...
site Asn-247–sialylated N-glycans serve as a brake on ligand-
stimulated signaling by hindering VEGF-mediated receptor
dimerization, activation, and downstream signaling. Con-
versely, in the absence of sialylated N-glycans at site Asn-247,
the extents of receptor dimerization, activation, and signaling
increase.

Treatment of VEGFR2 with PNGase F to remove N-linked
glycans increased ligand-induced receptor activation in vitro;
these effects were also observed in an in vitro kinase activity
assay. Site-directed mutagenesis of N-glycosylation sites within
Ig-like domains D2 and D3 in the extracellular domain of
VEGFR2 led to the identification of site Asn-247 as significant
in the hindrance of VEGFR2 activation in porcine and human
endothelial cell lines. Characterization of N-glycopeptides from
surface and nonsurface VEGFR2 via nUPLC-MS/MS enabled
the assignment of Asn-247–sialylated glycosyl forms in surface
VEGFR2. Enzymatic removal of sialic acids in WT and Asn-247
mutant VEGFR2 led to the identification of sialic acid modifi-
cations on Asn-247 N-linked glycans as important mediators
against VEGFR2 activation. Measurement of phosphorylated
Tyr-1175 on VEGFR2, an SH2-domain binding site, and phos-
phorylated PLCy1 and MAPK signaling molecules, confirmed
that site Asn-247 hindrance has downstream signaling impacts.
Finally, by measurement of VEGFR2 dimers and surface
levels of VEGFR2, we confirmed that Asn-247 glycans hinder
dimerization. In light of evidence that pro-inflammatory
cytokines increase the expression of α,6-linked sialic acid–
containing carbohydrate epitopes on the endothelial cell
surface while immunosuppressive cytokines reduce α,6-
linked sialic acid–containing carbohydrate epitopes (4, 6, 7),
we suggest that our results provide evidence of a mechanism
for cross-talk between inflammatory and angiogenic signal-
ning pathways.

How does N-glycosylation alter VEGFR2 dimerization and
signaling? VEGF ligand binding is known to bring two VEGFR2
monomers together to facilitate dimerization (53). Dim-
erization orients the kinase domains of each monomer, ena-
baling one receptor monomer to phosphorylate the other. The
in vitro kinase activity assay, by supplying ATP as a
substrate for the kinase at multiple concentrations, facili-
tates the measurement of how successfully VEGFR2 can ini-
tiate this autophosphorylation, with dimerization as a pre-
requisite for phosphorylation. While this was performed in
the absence of VEGF ligand, two receptor monomers must
still encounter each other in the proper orientation for one
to phosphorylate the other. We presume that because there
is no ligand present, the relief from “hindrance” observed in
the N247Q mutant VEGFR2 compared with the WT may be
(at least in part) due to altered interactions between the two
receptor monomers. The full nature of this interaction will
be the subject of future studies, although it may be due to 1)
repulsion between sialylated WT monomers with negative
charge arising from addition of sialic acid to the glycan at site
Asn-247, or 2) steric hindrance due to the addition of the
sialylated glycan.

Changes in VEGFR2 glycosylation may result in the develop-
ment of resistance to anti-angiogenic agents via galectin-medi-
ated ligand-independent activation of VEGFR2 (4, 7). Tumor
microenvironment–induced changes in VEGFR2 glycosylation
may also regulate angiogenic signaling by exhibiting or masking
receptor-binding sites for pro- or anti-angiogenic ligands, pro-
viding a functional link between glycan structure and angio-
genic signaling (7, 8). Previous studies of VEGFR2 by Croci et al.
(4) support the first model of RTK glycosylation, whereby lect-
tins are the primary mediators of glycosylation-dependent reg-
ulation. Our observations suggest that, in addition to the find-
ings reported by Croci et al. (4), a second mechanism exists, in
which changes in VEGFR2 glycosylation impact VEGFR2
ligand-dependent signaling directly, by altering receptor
dimerization. However, although our results show some simi-
larities to glycosylation-dependent regulation of EGFR, there
are also clear differences between the mechanisms by which
glycosylation impacts EGFR and VEGFR2 signaling. We
observed that, whereas sialylation of VEGFR2 at Asn-247 hin-
dered VEGFR2 signaling (similar to observations for EGFR),
treatment with fucosidases or inhibition of fucosyltransferases
with 2-fluorofucose did not appear to impact VEGFR2 activa-
tion, suggesting that fucosylated N-glycans do not play a direct
role in the regulation of VEGFR2 dimerization and signaling, in
contrast to their reported involvement in regulation of EGFR
dimerization.

Although sialylated N-glycans at site Asn-247 appear to play
a significant role in hindering VEGFR2 dimerization, activa-
tion, and signaling, it is likely that additional sites are involved,
as the magnitude change in Tyr-1054 phosphorylation between
PNGase F–treated versus untreated VEGFR2 was somewhat
higher than we observed during mutagenesis experiments that
targeted the D2 and D3 glycosylation sites. We suggest that
additional VEGFR2 N-glycosylation sites may be involved in
the observed effect. Now that we have determined that the Asn-
247 glycan has a structure-specific role in receptor activation
(whereas the glycans at Asn-145, Asn-160, and Asn-320 do
not), we have plans to examine the effects of modifying further
sites, but this is beyond the scope of this study. The outcome
should indicate whether general principles may govern which
glycosylation sites are likely to impact RTK dimerization and
signaling. Factors that may be relevant include the following:
(a) the solvent accessibility of glycosylation, a particular glyco-
sylation site, which likely serves as an indicator of how available
a glycan is to interact with other molecules; (b) the proximity
of a glycosylation site to the dimer interface or the interface
with another binding protein such as a ligand or lectin; and
(c) the ability of a specific glycosylation site to be processed
in the ER and Golgi to a form that lends the glycan a func-
tional outcome. Our characterization of surface and nonsur-
face VEGFR2 glycosylation at multiple sites revealed sites
that had the same glycans in both populations and sites
where glycan compositions differed between surface and
nonsurface populations. It is tempting to speculate that the
less processed sites (which share the same glycans between
surface and nonsurface populations) may have less relevance
and impact on receptor activation, due to their inability to
adapt to cellular conditions and stimuli and their reduced
exposure to potential interactors.

On the basis of the results that we report here, it appears that
the primary role of N-glycosylation at Asn-247 is to hinder full
activation of VEGFR2 when sialylated N-glycans are present, whereas other sites, including Asn-145, Asn-160, and Asn-320 in Ig-like domains D2 and D3, may either be neutral bystanders or perhaps some may carryers of N-glycans that serve as binding sites for lectins. Abrerrant protein glycosylation is one of the hallmarks of tumor cells, and oncosgenic Ras signaling can give rise to altered terminal sialylation of glycoproteins (61, 62). Pro-inflammatory cytokines in the tumor microenvironment lead to increased α2,6-linked sialylation of endothelial cell glycoproteins, whereas anti-inflammatory cytokines have the opposite effect, but the implications of these changes have not been well-explored (4). This suggests that there may be an important link between pathways that promote terminal sialylation and RTK signaling. Further studies will reveal how additional VEGFR2 sites in additional Ig-like domains contribute to ligand-dependent activation of VEGFR2 and whether glycans at Asn-247 sterically block dimerization. Normalization of tumor blood vessels is a promising strategy for treatment of cancer and may decrease metastasis, increase drug access into a tumor, and favor T-cell infiltration and immune therapy approaches. By illuminating the significance of VEGFR2 extracellular domain glycosylation and its role in dimerization, activation, and signaling, it is our hope that new strategies targeting VEGFR2 glycosylation may ultimately play a role in blocking the proliferation of tumor blood vessels to achieve these outcomes.

Experimental procedures
Reagents and antibodies

The following antibodies were used for Western blotting analyses: mouse monoclonal anti-α-tubulin (DM1A), rabbit monoclonal anti-phosphotyrosine 1175–VEGFR2 (19A10), phosphotyrosine 783–PLC-γ1, and anti-phospho-p44/p42 MAPK (threonine 202/tyrosine 204) (197G2) were all purchased from Cell Signaling Technology (Danvers, MA). A rabbit monoclonal antibody raised in-house against amino acids corresponding to the kinase insert of VEGFR2 was used for detection of VEGFR2 via Western blot analysis (63). Anti-Flk-1 (A-3) antibody coupled to agarose, anti-PLC-γ (1249), mouse anti-rabbit IgG-HRP, and rabbit anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Dallas, TX). Human VEGF-A was produced in our laboratory. Glycerol-free PNGase F, Q5 high-fidelity DNA polymerase, T4 DNA ligase, NotI-HF, Clal, and CutSmart buffer were obtained from New England Biolabs (Ipswich, MA). QIAquick gel extraction kit and the plasmid midi kit were obtained from Qiagen (Hilden, Germany). EZ-LinkTM sulfo-NHS-SS-biotinylation kit, NuPAGE™ 4–12% BisTris gels, NuPAGE™ MES SDS Running Buffer (10×), HiMark™ Pre-stained Protein Standard, Pierce ECL Western blotting substrate, and TOP10 Escherichia coli were purchased from Thermo Fisher Scientific (Wal-tham, MA).

Site-directed mutagenesis

Glycosylation site mutant VEGFR2 constructs were created using an overlap extension PCR strategy, as described previ-ously (16). The cDNA of the VEGFR2 extracellular domain was subcloned in the pCMV-SPORT6 vector and used as a template. To produce asparagine to glutamine mutations at the aforementioned sites, the following oligonucleotides corresponding to VEGFR2, and containing a single site mutation to convert Asn to Gln, were used: 5′-CACCCAGTTTTTTGCTTTGTTCGTT-3′ (for N145Q); 5′-GCAAAGAGACACTTGAGGTTTGAAT-3′ (for N160Q); 5′-TCTCGCTGTCATTTGTAAGACAAGTTT-3′ (for N247Q); and 5′-GCAAATGTCTTTGCTCTTGTATCAT-3′ (for N320Q).

For each construct, two fragments were created. The first fragment that spanned the region corresponding to the N terminus of VEGFR2, up to and containing the site of mutation, was produced with a NotI sense primer and an antisense primer corresponding to the sequence of VEGFR2 with the glycosylation site mutation, as listed above. The second fragment spanned the region corresponding to the site of mutation and extended to the C terminus of the VEGFR2 extracellular domain (sense), bound by a Clal site (antisense). PCR products were run on a 0.7% agarose gel, and DNA fragments were extracted using QIAquick gel extraction kit. Overlap extension PCR was performed by combining the fragment pairs for each mutant, with NotI sense and Clal antisense primers. Products were separated on a 0.7% agarose gel, extracted, and subjected to a restriction digestion with NotI-HF and Clal enzymes. Next, the fragments corresponding to the WT VEGFR2 extracellular domain or containing single glycosylation site mutations were ligated into a pLNCX2 vector (previously modified to remove HindIII and Clal sites) containing the transmembrane and cytoplasmic regions of VEGFR2 (15). VEGFR2 WT and mutant constructs (N145Q, N160Q, N247Q, and N320Q) are shown in Figs. S22–S26. The resultant mutations were verified by DNA sequencing.

Cell lines and retroviral transfection

Spontaneously immortalized PAE cells and HUVEC cells, expressing human telomerase (Tert) and lacking endogenous expression of VEGFR2, were transfected with WT or glycosylation-site mutant murine VEGFR2 using constructs described above. Briefly, TOP10 E. coli cells were transformed with WT mFlk-1, mFlk-1 N145Q, mFlk-1 N160Q, mFlk-1 N247Q, or mFlk-1 N320Q constructs, followed by ampicillin selection and plasmid isolation via a plasmid midi kit (Qiagen, Hilden, Germany). Purified plasmids were used to transfect 293GPG cells for production of high-titer virus particles (64). Equal numbers of colony-forming units from the concentrated virus were used to transfect PAE and HUVEC(Tert) cells. Cells were subsequently selected with G418 (geneticin). Following selection, cell lines derived from the PAE parent cell line were grown in Dul-becco’s modified Eagle’s medium with 10% FBS and penicillin/streptomycin. Cell lines derived from the HUVEC(Tert) parent cell line were passaged with RPMI 1640 medium with 10% FBS and penicillin/streptomycin.

Ligand stimulation and Western blot analysis

Immortalized PAE and HUVEC(Tert) cell lines were grown in sparse conditions (>70% confluence) in 10% FBS and serum-starved overnight (16–18 h.) in serum-free medium. Cells were
left resting or treated with VEGF (100 ng/ml) for 5, 10, or 30 min at 37 °C. Cells were washed once with ice-cold H/S buffer (25 mM HEPES, pH 7.4, 150 mM NaCl) and lysed with Extraction Buffer (EB) containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, and 20 µg/ml aprotinin. For Western blot analysis of whole-cell lysate, lysate was spun at 14,000 relative centrifugal force for 15 min at 4 °C; supernatant was transferred to new tubes; an appropriate volume of 5× sample buffer containing β-mercaptoethanol was added; and the mixture was heated at 95 °C for 5 min. Whole-cell lysates were resolved either on 7.5% NuPAGE or 4–12% BisTris SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% (w/v) nonfat dry milk, and 0.05% Tween 20 and then incubated for 1 h with primary antibody diluted in the blocking solution. Membranes were washed, incubated for 1 h with HRP-conjugated secondary antibodies, washed again, and processed with Pierce ECL Western blotting substrate. To blot for total VEGFR2, membranes were stripped by incubating with 6.25 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 30 min, washed, and re-probed.

**On-cell PNGase F, neuraminidase, and fucosidase treatment**

Live cells were treated with PNGase F to remove N-linked glycans according to a previously published protocol (65), with modifications. Briefly, cells at 70% confluence were serum-starved overnight in serum-free medium, followed by de-N-glycosylation with PNGase F (10,000 units/ml) for 4 h at 37 °C, followed by washing three times with cold serum-free medium, and addition of VEGF (100 ng/ml) for 0, 5, 10, or 30 min. After cells were treated with VEGF, they were lysed as described above. For neuraminidase and fucosidase experiments, the same procedure was used, except that cells were incubated with 1300 units/ml enzyme for 1.5 h at 37 °C followed by 30 min at 4 °C.

**In vitro kinase assay**

The kinase activity of VEGFR2, with and without PNGase F treatment, was assayed as described previously (66), with modifications. Briefly, VEGFR2/PAE cells were treated with 50 µM MG132 for 24 h prior to cell lysis, followed by immunoprecipitation of VEGFR2 and two wash cycles with PAN buffer containing 10 mM PIPES, pH 7.0, 100 mM NaCl, 20 µg/ml aprotinin, and 0.5% Nonidet P-40. Equal amounts of VEGFR2 were used for control (untreated, with identical buffer conditions) and PNGase F treatment (500 units of PNGase F), and the immunoprecipitates were incubated at 37 °C for 1 h. Control and PNGase F–treated samples were divided equally into tubes, and an equal volume of 2X kinase buffer was added for a final concentration of 10 mM MgCl2, 1 mM DTT, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and then 0, 0.1, or 1 mM ATP was added and incubated for 15 min at 37 °C. The reaction was stopped by addition of an equal volume of SDS sample buffer. VEGFR2 was resolved on a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane, and Western blot analysis was performed with anti-phospho-Tyr-1054–VEGFR2 and anti-VEGFR2 (total) antibodies.

**Cell-surface biotinylation, immunoprecipitation, and mass spectrometry analysis of VEGFR2**

To isolate surface and nonsurface fractions of VEGFR2, VEGFR2/PAE cells were grown to 70% confluence, washed, labeled with membrane-impermeable EZ-Link sulfo-NHS-SS-biotin, and lysed. Biotinylated and unlabeled fractions were separated using NeutrAvidin-agarose, according to the manufacturer’s protocol. Western blottings were performed to confirm the successful separation of labeled and unlabeled fractions, and VEGFR2 was immunoprecipitated from the unused portion of each fraction. Separately, VEGFR2 was immunoprecipitated from unlabeled lysates, and the high- and low-molecular-weight bands were resolved on a 7.5% SDS-polyacrylamide gel. All VEGFR2 samples were subsequently treated with trypsin and analyzed via nUPLC-MS/MS to assess VEGFR2 site-specific N-glycosylation, as described previously (47, 67).

Briefly, VEGFR2 glycopeptides were enriched, separated, and analyzed using a 6550 Q-TOF MS with a 1200 series nanoflow HPLC-Chip-ESI source fitted with a custom HPLC-Chip with 360 nl of TSK gel amide-80, 5-µm trapping and enrichment column, and a 150-µm × 75-µm Polaris C18-A 3-µm analytical column (all from Agilent Corp., Santa Clara, CA). The 6550 Q-TOF mass spectrometer was operated in positive mode using the high-resolution, extended dynamic range (2 GHz) setting. Spectra were collected in the profile mode. The mass measurement error for all peaks with signal-to-noise ratio of >10 was within 5 ppm. To assign glycopeptide tandem mass spectra, nUPLC-MS/MS data were processed using Byonic version 2.13.17 (Protein Metrics, San Carlos, CA), with the murine VEGFR2 sequence from the Reviewed UniProtKB/Swiss-Prot protein sequence database (last modified March 13, 2018).

For targeted analyses of VEGFR2 glycopeptides, samples were analyzed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoACQUITY UPLC system (Waters) and a TriVersa NanoMate (Advion). For chromatographic separation, a nanoACQUITY UPLC Symmetry C18 trap column (100 Å, 5 µm, 180-µm × 20-mm, Waters) was used for trapping, and an ACQUITY UPLC Peptide BEH C18 nanoACQUITY column (130 Å, 1.7 µm, 150-µm × 100-mm, Waters) was used for separation. The peptide trapping step was performed at 4 µl/min for 4 min with 1% acetonitrile and 0.1% formic acid (solvent A). Following the trapping step, peptides were separated on the analytical column according to the following conditions: 0–1 min, 2% B; 1–3 min, 2–5% B; 3–43 min, 5–40% B (solvent A: 1% acetonitrile and 0.1% formic acid in water; solvent B: 99% acetonitrile, 1% water, and 0.1% formic acid). MS scans were acquired with the following settings: 70,000 resolution at m/z 400, scan range m/z 370–2000, 1 microscan/MS, AGC target 1 × 106, and a maximum injection time of 50 ms. MS2 scans were acquired with the following settings: 17,500 resolution at m/z 400, AGC target of 1 × 106, maximum injection time of 150 ms, isolation window of 2.0 m/z, isolation offset of 0.4 m/z, exclusion of charge states 1 and >8, and
underfill ratio of 1.2%. A list of targets was provided, and targeted parallel reaction monitoring (PRM) with alternating normalized collision energies of 30, 35, and 40% were used within a single run. Profile data were recorded for MS and MS2 scans.

For relative quantification of glycopeptides derived from surface and nonsurface VEGFR2 and neuraminidase-treated VEGFR2, samples were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an ACQUITY UPLC M-Class system (Waters) and a TriVersa NanoMate (Advion). For chromatographic separation, a nanoEase Symmetry C18 UPLC trap column (100 Å, 5 µm, 180-µm × 20-mm, Waters) was used for trapping, and a nanoEase MZ HSS C18 T3 UPLC column (100 Å, 1.8 µm, 75-µm × 100-mm, Waters) was used for separation. The peptide trapping and separation were performed as described above for peptide/glycopeptide analysis. MS scans were acquired with the following settings: 120,000 resolution at m/z 400, scan range m/z 500–1200, 4 microscans/MS, RF lens set to 30%, AGC target 1 × 106, and a maximum injection time of 100 ms.

**VEGFR2 dimerization**

VEGFR2/PAE and VEGFR2 N247Q/PAE dimer formation was assessed to determine whether changes in N-glycosylation impact the level of ligand-mediated receptor dimerization. In response to ligand-mediated dimerization, spontaneous disulfide bond formation occurs in the cytoplasmic domain of VEGFR2, and this stabilizes the VEGFR2 dimer (51). VEGFR2/PAE and VEGFR2 N247Q/PAE cells were serum-starved overnight, followed by VEGF ligand (100 ng/ml) stimulation for 0, 5, 10, or 30 min. Cells were washed and lysed, and whole-cell lysates were divided in half and treated either with nonreducing SDS sample buffer (without β-mercaptoethanol) and heated at 55 °C for 5 min, or with sample buffer containing β-mercaptoethanol and heated at 95 °C for 5 min. To resolve dimerized, disulfide-stabilized VEGFR2 from VEGFR2 monomer, lysates were resolved on a 7.5% SDS-polyacrylamide gel, followed by transfer to a PVDF membrane and detection with an anti-Flk1 antibody.

**Statistical analyses**

Summary statistics, including the mean and standard deviation (S.D.), are presented. Two-tailed t tests were used to calculate p values; the Data Analysis tool within Microsoft Excel 2013 was used for statistical calculations. All experiments were performed ≥3 times. The numbers of replicates performed for each experiment are listed in each figure. Significant and non-significant p values are reported in each figure. Western blots were quantified with ImageJ software and normalized to total VEGFR-2 when appropriate.

**Data availability**

The datasets generated and/or analyzed during this study are available from the corresponding author on reasonable request. Mass spectral data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with accession PXD012629.

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