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Ectodomain cleavage of FLT1 regulates receptor activation and function and is not required for its downstream intracellular cleavage

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Abstract

Flt1 is a cell surface VEGF receptor which is cleaved to release an N-terminal ectodomain which binds VEGF and PlGF and can antagonize the effects of VEGF in the extracellular milieu. To further evaluate Flt1 processing we expressed tagged Flt1 constructs in HEK293 and COS7 cells where we demonstrate, by deletion mapping, that the cleavage site is immediately adjacent to the transmembrane domain (TMD) between residues 759 and 763. Cleavage reciprocally regulates free VEGF in conditioned media and we show that the cleavage site is also transferable to another transmembrane receptor. A second cleavage event downstream of the ectodomain cleavage releases a cytosolic C-terminal Flt1 fragment and this intracellular cleavage of Flt1 is not catalyzed or regulated by the upstream ectodomain cleavage since abolition of the ectodomain cleavage has no impact on the downstream cleavage event. The downstream cleavage event is not susceptible to γ -secretase inhibitors and overexpression of presenilin 1, the catalytic subunit of γ -secretase did not change the downstream intracellular cleavage event. Furthermore, this cleavage did not occur via a previously published valine residue (767V) in the TMD of Flt1, indicating the existence of another cleavage pathway. We tested the impact of the ectodomain cleavage on p44/42 MAP kinase activation and demonstrate that compared to wild type Flt1, cleavage resistant Flt1 constructs failed to stimulate p44/42 MAP kinase activation. Our results indicate that Flt1 ectodomain cleavage not only regulates the availability of free VEGF in the extracellular milieu but also regulates cellular signaling via the ERK kinase pathway.

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Keywords

proteolytic cleavage; soluble receptor; MAP kinase

Introduction

FLT1, also known as VEGFR1, is one of the two principal cell surface receptors for VEGF and is critically important for angiogenesis not only in development but also during pregnancy and following injury (1, 2). FLT1 and KDR also known as VEGFR2 are type 1 transmembrane proteins with an extracellular N-terminal region that includes the ligand binding domain, a single transmembrane domain and an intracellular C-terminal region that contains a split tyrosine kinase domain (3, 4). VEGF and a related growth factor PlGF bind VEGF receptors as a homodimer or heterodimer leading to receptor tyrosine phosphorylation and downstream signaling including the activation of protein kinase C (PKC) and MAP kinases. The activity of the VEGF receptors can also be regulated by the presence of naturally occurring receptor antagonists. In this regard, several truncated Flt1 variants bind VEGF and PlGF with high affinity reducing free ligand and thus inhibiting receptor function (5). Two of the soluble Flt1 (sFlt1) variants are transcriptionally derived and prematurely terminate by alternate splicing and utilization of upstream polyadenylation sites to yield secreted proteins that lack the transmembrane and C-terminal domains (6–9). FLT1 is also proteolytically cleaved close to the transmembrane domain by ADAM metalloproteases to release the N-terminal fragment into the extracellular milieu (10). Cleaved Flt1 (cFlt1) like sFlt1 contain the VEGF binding domain and serves as a decoy receptor to reduce VEGF and PlGF access to its cognate cell surface receptors and thus function as VEGF and/or PlGF antagonists.

Proteolytic cleaving of surface proteins is now widely recognized as a mechanism for the release of protein fragments that serve a wide variety of purposes (11, 12). In some instances as with Flt1, the release of a soluble receptor antagonist is one mechanism to regulate VEGF function in an autocrine, paracrine or endocrine fashion. In other situations, proteolytic cleaving is used to release proligands such as proHB-EGF and proTGF- α as soluble agonists, or to increase circulating cytokines such as TNF- α or cell adhesion molecules such as selectins and cadherins (13). One of the common 'shedases' are metalloproteases of the ADAM superfamily and individual ADAMs can cleave multiple substrates and the same substrate can be cleaved sometimes by more than one ADAM protease (12). The extracellular cleavage of membrane proteins do not appear to be determined by a unique signature or common motif within the target protein although the cleavage site is usually close to the TMD and it is unclear if secondary structures in this area or the proximity to the TMD are key determinants of cleavage.

Many extracellular cleavage events are accompanied by a downstream cleavage event that occurs within or just beyond the TMD which releases a fragment internally. This process, called regulated intramembrane proteolysis (RIPS) seems to follow the upstream cleavage event (14–16). The internally released fragments may traffic to the nucleus or other intracellular organelles and be involved in transcription or in cellular signaling or be a

mechanism to stimulate target protein release, terminate protein function or to effect its degradation. The enzymes that catalyze RIPS are called intramembrane-cleaving proteases (iCLIPS) and generally belong to one of three enzyme families. These are the aspartyl proteases like γ -secretase, the zinc metalloproteinase site-2 proteinase and serine proteases of the rhomboid family (16–18).

In this manuscript, we further explore the cleavage of Flt1. We identify the site of ectodomain cleavage and demonstrate a second cleavage event that releases a cytosolic fragment. Remarkably, the downstream cleavage event can occur without the preceding upstream cleavage challenging the dogma that ectodomain cleavage is a prerequisite for the intracellular cleavage. This downstream cleavage does not appear to be γ -secretase dependent. We also show that cleavage resistant Flt1 mutants demonstrates lower p44/42 MAP kinase activation compared to wild type FLT1 suggesting that cleavage regulates receptor activation and signaling.

Materials and Methods

Heparin, phorbol 12-myristate 13-acetate (PMA), L-685,458, DAPT and Suberic acid bis (N-hydroxy-succinimide ester, DSS) were purchased from Sigma-Aldrich (St. Louis, MO) and Compound E was from EMD Millipore (Billerica, MA). Human VEGF ELISA Kit was obtained from R&D Systems (Minneapolis, MN). Antibodies: HA (Y-11), alkaline phosphatase (sc-28904), α -Tubulin (sc-8035), HSP90 (sc-69703), EGFR (sc-03), Presenilin 1 (sc-7860), HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgM were from Santa Cruz Biotechnology (Santa Cruz, CA). p44/42 MAPK (9102), phospho-p44/42 MAPK (Thr202/Tyr204, 9101) Ab and HRP-conjugated goat anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA), anti-myc antibody (R950-25) was from Life Technologies (Carlsbad, CA), anti-Flag antibody (F-3165) was from Sigma-Aldrich and turbo-GFP Ab (TA150041) was from OriGene Technologies (Rockville, MD).

Cell culture

Human embryonic kidney cell line HEK293 and African green monkey fibroblast cell line COS7 were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. Cultured cells were maintained at 37°C in a humidified air atmosphere of 5% CO₂.

Expression vectors

The expression vector for presenilin 1 was a gift from G. Thinakaran (University of Chicago).and Notch1 (#41728) was from Addgene (Cambridge, MA). Expression vectors for HA-VEGFR1-Myc-Flag, Flt1 TK and KDR-tGFP have been described before (10, 19). Flt1 729-758 and 699-758 mutants were created via an overlapping 2-step PCR cloning process with the primers mentioned below. Similarly chimeric receptor HA-VEGFR1/KDR-tGFP composed of the extracellular region and TMD of HA-VEGFR1 was fused to the intracellular domain of KDR-tGFP via overlapping 2-step PCR cloning process with the primers below. All other amino acid deletion or substitution constructs were created by

QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies, La Jolla, CA). All constructs were verified by Sanger sequencing.

Primer sequences to generate Flt1 729-758 mutant:

Flt1_HindIII_F: ACATAAGCTTTTATATCACAGATGTGCCAA

Flt1_Int_R: GTGATCAGGACACCTTCATCCTCTTCTGTGACT

Flt1_Int_F: AAGGTGTCCTGATCACTCTAACATGCACCTGTG

Flt1_HindIII_R: CGCAAAGCTTTCGCTGCTGGT

Primer sequences to generate Flt1 699-758 mutant:

Flt1_HindIII_F: ACATAAGCTTTTATATCACAGATGTGCCAA

Flt1_Int_R2: GTGATCAGGTTGTTTAAACCAAGTGATCTGAGGC

Flt1_Int_F2: AAAACAACCTGATCACTCTAACATGCACCTGTG

Flt1_HindIII_R: CGCAAAGCTTTCGCTGCTGGT

Primer sequences to generate HA-VEGFR1/KDR-tGFP chimeric receptor:

HA-Flt1_F: ATCCGGTACCGAGGAGATCTGCC

Flt1_R: GCCCGCTTGATAAAGAGGGTTAATAGGAGCCAG

KDR_F: TCTTTATCAAGCGGGCCAATGGAGGGGA

KDR-tGFP_R: GGCCGTTTAAACTCTTTCTTCACCGGC

Transient transfections, preparation of cell lysates and subcellular fractions

HEK293 or COS7 were transiently transfected with plasmid vectors using Lipofectamine 3000 from Life Technologies (Grand Island, NY). Transfected cells were serum starved overnight and then the cultured cells were exposed to specific reagents in serum and antibiotic-free medium for various times. When completed, conditioned media was collected and monolayers were washed with ice cold PBS and kept frozen until whole cell protein lysates were prepared for western blotting. Conditioned media was concentrated using Amicon Ultra centrifugal filters from EMD Millipore (Billerica, MA) and cell lysates were solubilized in RIPA buffer (Pierce, Rockford, IL) containing protease inhibitor cocktail from Roche Applied Science (Indianapolis, IN). In some experiments, cells were trypsinized, counted and then an equal number of cells were processed for cytosolic and membrane protein fractions using ProteoExtract® Subcellular Proteome Extraction Kit (EMD Millipore, Billerica, MA). In other experiments cell surface proteins were extracted with the Pinpoint™ Cell Surface Protein Isolation Kit (Pierce, Rockford, IL).

Chemical Crosslinking of Receptor Dimers

COS7 cells expressing Flt1 or Flt1 754-763 were incubated in serum-free high glucose DMEM containing 50mM Hepes (pH 7) at 4°C for 90 min. After incubation, cells were washed 3X with PBS at room temperature and then 0.5 mM disuccinimidyl suberate (DSS) in PBS was added for crosslinking. Crosslinking reaction was quenched after 30 min by

replacing with 10 mM Tris buffer. Cells were then washed multiple times with ice-cold PBS and whole cell lysates prepared in RIPA buffer for western blotting.

Western blotting and ELISA

For western blotting equal amounts of whole cell lysate, concentrated conditioned media, cytosol or membrane fractions or surface proteins were heated with 2X Laemmli sample buffer (3% sodium dodecyl sulfate, 12% glycerol, 50mM Tris, pH 6.8 and 80mM dithiothreitol) to 95°C for 5min and then resolved by SDS-PAGE. Resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA). PVDF membranes were blocked in 5% milk for at an hour at room temperature then incubated sequentially with primary antibody overnight and HRP-conjugated secondary antibody for one hour. Signals were detected with SuperSignal West Pico, Dura or Femto Chemiluminescent Substrate from Thermo Fisher Scientific (Rockford, IL) and the image was captured and quantitated using VisionWorksTMLS image acquisition and analysis software and the EC³ imaging system from UVP LLC (Upland, CA). PVDF membranes were stripped with 0.2M NaOH for 30 min at room temperature for repeated blotting.

Quantitation of VEGF in conditioned media was performed by ELISA (Quantikine human VEGF, R&D Systems, Minneapolis, MN).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation. The analysis were performed with a Student's t-test or one-way analysis of variance (ANOVA) where applicable, using SigmaPlot[®] 12 (San Jose, CA). Statistical significance is indicated in each figure when the p value was at least less than 0.05 ($p < 0.05$).

Results

In previous work we have shown that PKC activation stimulates the cleavage of an ectodomain of Flt1 that is mediated via ADAM10 and ADAM17 (10). The cleaved ectodomain shares substantial identity with transcribed forms of Flt1 that lack both the transmembrane domain and the intracellular portion, and are secreted and are collectively known as soluble Flt1 (sFlt1). Cleaved Flt1, like sFlt1, is heavily glycosylated, can bind VEGF and PlGF and can function as a VEGF antagonist in the extracellular milieu (19). The size of the Flt1 ectodomain indicates a cleavage site close to the predicted TMD. This cleavage leaves the C-terminal portion still membrane attached, although others have identified an ~80 kDa cytosolic fragment possibly arising from the separate action of γ -secretase at a valine residue at position 767 within the TM domain (20, 21).

To determine if there were two separate cleavage events we expressed Flt1 with an N-term HA tag and a C-term Flag tag in HEK293 cells and stimulated cleavage with PMA. Conditioned media, membrane and cytosolic fractions were collected and blotted for the respective epitope tagged fragments (Figure 1A). We identified a ~110 kDa HA fragment in conditioned media, the full length FLAG tagged ~ 180 kDa FLT1 and a C-terminal ~65 kDa FLAG tagged fragment in membrane fractions and a ~ 60 kDa FLAG tagged fragment in the soluble fraction (cytosol). We saw similar results with Flt1 expression in COS-7 cells

(Figure 1B). These findings are consistent with two cleavage events wherein the ~110 kDa N-terminal fragment (NTF) in conditioned media and the 65 kDa C-terminal fragment (C-terminal fragment 1 – CTF 1) in membrane fractions are products generated by the upstream cleavage and the ~60 kDa cytosolic FLAG tagged fragment (C-terminal fragment 2 – CTF 2) represents another cleavage event occurring intracellularly. Together this dual cleavage could be an example of regulated intramembrane proteolysis (RIPS) with the intracellular cleavage step coming after and dependent on the extracellular ADAM protease-mediated cleavage, analogous to a number of other type 1 membrane proteins (14, 16). Since γ -secretase had been previously reported to cleave FLT1 at the downstream site, we tested the effect of γ -secretase inhibitors, L685,458, compound E and DAPT. If γ -secretase is active the expected findings are an increase in the 65 kDa fragment in membrane fractions reflecting an accumulation of the upstream cleaved product and a reduction in the ~60 kDa fragment in the cytosol. We see a significant increase in the ~65 kDa cleavage product, but were unable to find a significant effect of these compounds on the ~60 kDa cleavage product (Figure 1, panels C and D) suggesting that this cleavage was mediated by a different enzymatic activity.

To further explore cleavage we began a series of experiments to identify the extracellular cleavage site by creating a variety of deletion and substitution mutants that altered residues in the extracellular region adjacent to the predicted TMD. The TMD of FLT1 is thought to extend from 759 to 780 (www.uniprot.org). Removal of a 30 AA region corresponding to residues 729 to 758 in human Flt1 (729-758) increased expression of the Flt1 mutant (1.81 + 0.66 fold; n=5, p<0.05) compared to wild type Flt1 with a corresponding increase in the cleaved fragments NTF and CTF1. With the removal of an additional 30 AA (residues 699 to 758, 699-758) there was no longer an impact on the abundance of the full-length mutant (Figure 2A and 2C). When an additional 5 residues, LITLT, abutting on the transmembrane domain were also deleted (65 residues, 699-763) there was a substantial reduction in ectodomain cleavage and this inhibition of cleavage was seen by deleting just the last 10 residues including the downstream LITLT (754-763). The reduced cleavage was not attributable to lack of expression or to relative instability of the mutant protein as the uncleaved mutants were expressed in similar amounts to the unmodified full-length protein in cell lysates (Figure 2A). To determine the impact of the selective mutation that diminished or eliminated the ectodomain cleavage on the downstream cleavage event, we separated membrane and cytosol fractions. In the Flt1 mutant construct where ectodomain cleavage is affected, the reduction in ectodomain was matched by a corresponding loss of the C-term product of that upstream cleavage. Surprisingly the C-terminal downstream cleavage site was not affected indicating that the downstream cleavage event is not dependent on the ectodomain cleavage event (Figure 2A and 2C) and is separately regulated from the extracellular cleavage event.

To determine if the residues LITLT contained the actual cleavage site we compared a mutant where these residues were deleted (759-763) with the mutant where an additional 5 residues N-terminal to these were removed (754-763). Both constructs had near complete and similar reduction in ectodomain cleavage (Figure 3A and 3B). The presence of low-level cleaved product with 759-763 and 754-763 suggests that there may be secondary cleavage sites that contribute in a small way to the total ectodomain generated. We also

generated 3 additional mutants to attempt to identify important residues within LITLT (Figure 3A and 3B). In one the first 2 residues LI were mutated to AA and the remaining residues TLT were deleted (AA 761-763); in another 3 of 5 residues (761-763) were deleted, and in the 3rd, a single residue at position 759 was deleted (759). AA 761-763 had a marked reduction in cleavage whereas 761-763 alone had no impact on cleavage. These experiments suggest that residues LI at 759–760 are most important for cleavage. Deletion of 759 alone had no impact on cleavage and although it appeared to affect the abundance of full length Flt1 this was not significant (0.67 ± 0.27 , $n=5$, $p > 0.05$).

We noted that all the mutations that affected upstream cleavage appeared to have no impact on the downstream cleavage event, thereby clearly dissociating these two cleavage events. Consistent with the lack of an effect of γ -secretase inhibitors on the downstream cleavage event, the dissociation of the cleavage events in Figures 2 and 3 also suggest an intracellular cleaving protease different from γ -secretase. We also note that the residues LITLT, that contains the upstream cleavage site, are perfectly conserved through evolution from birds to man (Figure 3C).

To further verify that some of the mutants had a major impact on the abundance of the cleaved NTF and to demonstrate that these cleaved fragments can reduce free VEGF, we tested conditioned media from each of these Flt1 constructs for its ability to bind VEGF in a VEGF ELISA. This ELISA measures free VEGF and not that bound to soluble receptors such as sFlt1 or cFlt1 (19, 22). We demonstrate that constructs that were efficiently cleaved substantially reduced free VEGF while those that were resistant to cleavage had a much smaller impact on VEGF binding (Figure 4A).

We have previously shown that the extracellular cleavage of Flt1 is mediated via the metalloproteases ADAM10 and 17 and the appearance of the cleaved ectodomain in conditioned media is consistent with the known effect of ADAMs to cleave cell surface proteins (10). To verify that constructs with reduced or absent cleavage were still expressed at the cell surface and therefore available for metalloprotease cleavage, we performed surface biotinylation experiments and confirmed that mutant constructs were expressed appropriately (Figure 4B). We then looked at receptor dimerization for 754-763, one of the cleavage resistant mutants, using the crosslinker DSS and demonstrate that a mutation close to the TMD that inhibits cleavage does not interfere with dimerization (figure 4C).

To verify that the sequence LITLT was sufficient to regulate cleavage and that no cytosolic residues were necessary, we transferred the region containing the FLT1 cleavage site to another type 1 transmembrane protein, KDR. We created a chimeric molecule containing the extracellular N-terminal domain and TMD of Flt1 and the cytosolic C-terminal domain of KDR. We demonstrate cleavage of the Flt1-KDR hybrid with the appearance of an N-terminal fragment in conditioned media (Figure 4D). No cleaved fragment was seen with wild type KDR, indicating that the primary cleavage site within the FLT1 extracellular domain was responsible for the cleavage seen with the FLT1-KDR hybrid. Deletion of the same 5 or 10 residues (759-763 and 754-763) in the EC domain of the hybrid substantially diminished cleavage of the hybrid just as we had demonstrated with Flt1, further confirming the role of this subsequence in Flt1 cleavage. These domain swapping

experiments also indicate that cytosolic sequences within Flt1 do not regulate extracellular cleavage. We cannot exclude the possibility that cytosolic sequences within KDR are capable of regulating extracellular cleavage although it would be unlikely that KDR regulates the cleavage of FLT1 at the very same site.

The intracellular cleavage that occurs downstream of the metalloprotease cleavage in Flt1 does not appear to require the upstream cleavage event unlike what has been reported for other type 1 transmembrane proteins (14, 16). Since all previous examples of the sequential dual cleavage process in type 1 membrane proteins have been via γ -secretase, our findings would argue that the intracellular or downstream cleavage of Flt1 is γ -secretase independent and another intramembrane cleaving protease (iCLiP) is probably involved. We then tested the effect of overexpression of wild type presenilin 1, the catalytic subunit of γ -secretase and could not demonstrate an impact on the downstream Flt1 cleavage event (Figure 5A and 5B). We used Notch as a positive control for presenilin action and verified that presenilin 1 efficiently cleaved its known substrate (Figure 5C) (23). It is important to note that others have reported that γ -secretase cleaves Flt1 at the Valine at position 767 (21). We mutated this residue to an alanine (V767A) and were unable to show any impact of this mutation on the intracellular cleavage event (Figure 5D and 5E). In any case it seems unlikely that the downstream cleavage site would be at V767 as that would place it too close to the upstream cleavage site (759-763) yet our data shows that the upstream and downstream cleaved C-term fragments can be resolved easily by size on an SDS-PAGE gel indicating that it is likely further downstream (see Figure 1A). Together, these data argue that the intracellular cleavage is mediated by an iCLiP other than γ -secretase.

We then looked at the impact of Flt1 cleavage on downstream signaling, specifically at p44/p42 MAP kinase activation. When expressed in HEK293 cells, Flt1 stimulated p44/42 MAP kinase phosphorylation (Figure 6A and 6B). Compared to Flt1, 759-763, 754-763 and 699-763 showed lower p44/42 phosphorylation in HEK293 cells. As expected CTD, an Flt1 construct that lacks the entire C-terminal domain showed minimal p44/42 phosphorylation and functioned as a dominant negative protein reducing basal p44/42 activation. We saw no effect on total MAPK abundance with any of the constructs (Figure 6C). Our results indicate that cleavage not only regulates the availability of free VEGF in the extracellular milieu but also regulates downstream signaling via the ERK kinase pathway.

Discussion

FLT1, like many other type 1 membrane proteins appears to be subject to ectodomain cleavage and a downstream intramembrane or cytosolic cleavage event. We have previously shown that the ectodomain cleavage does not require the presence of the intracellular domain suggesting that receptor activation and downstream signaling is not a prerequisite for cleavage. Ectodomain cleavage of membrane proteins can serve many functions including the release of a bioactive peptide or trigger a downstream intramembrane or cytosolic cleavage event which releases an intracellular fragment. The ectodomain can function in an autocrine, paracrine or endocrine fashion to antagonize or stimulate cellular events. As an example the ligand EGF is released by ectodomain cleavage from the cell surface where it is expressed as a type 1 membrane protein, proEGF (24). Similarly the intracellular fragment

can traffic to the nucleus or another intracellular organelle to serve a new function. For example, binding of ligand to Notch releases an intracellular domain that translocates to the nucleus where it functions as a transcription factor (25). Alternatively, one or both of these cleavage events serve to terminate the function of the protein from which these domains are cleaved. We have previously shown that FLT1 ectodomain cleavage results in the release of a soluble VEGF antagonist that regulates the abundance of free VEGF in the extracellular milieu (19). We also demonstrated that the ADAM metalloproteases and PKC activation stimulates FLT1 cleavage and that co-expression of KDR reduced the cleavage of FLT1 (10).

Given the importance of Flt1 cleavage in regulating extracellular and intracellular events we made a series of mutations to map the ectodomain cleavage site and to determine the impact of ectodomain cleavage on the intramembrane cleavage event. We identified a cleavage site very close to the transmembrane domain. Deleting a 5 AA motif containing the cleavage site resulted in loss of cleavage manifest both in the reduced N-terminal fragment in conditioned media and in reduced C-terminal fragment within membrane fractions (CTF 1). We verified that the lack of cleavage was not because the deletion interfered with stability, surface expression, VEGF binding or ability to dimerize. We then discovered that the upstream cleavage is not required for the downstream cleavage event as the abundance of the cytosolic 2nd fragment (CTF2) was unchanged with mutations that abolished upstream cleavage.

Previously it has been reported that the downstream cleavage event is mediated via γ -secretase (21). We were unable to demonstrate an effect on the downstream cleavage event with γ -secretase inhibitors or by overexpression of presenilin 1. We also found that mutation of a previously reported residue in the TMD of FLT1 that is the reported site of the downstream cleavage via γ -secretase action, did not have an impact on cleavage. This is not surprising since the previously reported residue is actually at the 5' end of the TMD near the extracellular/membrane interface which would not be predicted to release a cytosolic fragment untethered to the cell membrane. In fact, most PS- γ -secretase substrates have a cleavage site very close to the membrane/cytosol interface (26, 27)

As stated earlier, the released ectodomain of FLT1 can function as a soluble VEGF antagonist. We wondered if the cleavage may also impact receptor activation and signaling. We could not clearly identify Flt1 phosphorylation but did see an impact on Flt1 mediated p44/42 MAPK activation. While Flt1 increased MAPK activation each of the cleavage resistant mutants appeared to abolish the FLT1 effect. As expected, a C-terminal deleted form of FLT1 reduced basal MAPK signaling probably by functioning as a dominant negative by competing with native FLT1 or KDR receptors present in HEK293 cells. The lack of MAPK activation was not secondary to poor expression or localization of the mutant protein as we demonstrated that these constructs were expressed at the cell surface and were able to hetero-dimerize.

In conclusion we identify the metalloprotease mediated ectodomain cleavage site for FLT1 and establish that ectodomain cleavage is not necessary for the downstream intracellular cleavage event. Our findings also demonstrate that, in addition to increasing the abundance of a soluble VEGFR in the extracellular milieu, cleavage regulates receptor activation and downstream signaling.

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Highlights

Flt1 cleavage releases an N-terminal ectodomain which antagonizes the effects of VEGF

The Flt1 cleavage site is adjacent to the TMD between residues 759 and 763.

This cleavage site is not dependent on Flt1-specific intracellular residues.

A second downstream cleavage is not regulated by the upstream ectodomain cleavage.

Ectodomain cleavage regulates cellular signaling via the p42/44 MAP kinase pathway.

Figure 1.

The cleavage of FLT1 and the effect of γ -secretase inhibitors. Panel A and B: HEK293 (left) and COS7 cells (right) were transiently transfected with HA and Flag-tagged Flt1 or empty vector (pcDNA3) and treated overnight with 30 nM PMA and 10 U/ml of heparin. Conditioned media (CM), membrane and cytosolic fractions were probed with HA and Flag antibodies as indicated. Full length Flt1 (FL Flt1), the N-terminal fragment (NTF) and the C-terminal fragments (CTF1 and CTF2) are seen in relevant fractions as are some non-specific bands (NS). Schematic between depicts the domain structure of Flt1 with an extracellular domain (ECD), the transmembrane domain (TMD) and the C terminal fragments, CTF1 and CTF2. Panel C and D: HEK293 cells transiently expressing HA and Flag-tagged Flt1 were treated with γ -secretase inhibitors, compound E (10 μ M), L686,458 (5 μ M) and DAPT (10 μ M) for 24 hours. Various cellular fractions were resolved by SDS-PAGE and probed for NTF, CTF1 and CTF2 by immunoblotting with the indicated antibodies. A representative immunoblot is shown in B and pooled CTF data quantified by densitometry is shown in panel C. mean $\pm$ SD, n=3, *p< 0.05.

Figure 2.

Mapping the ectodomain cleavage site of FLT1. Panel A, B and C: HEK293 cells were transiently transfected with pcDNA3, FLT1 or various FLT1 deletion constructs together with a vector expressing secreted alkaline phosphatase (alk phos) and various fractions resolved by SDS page and probed with the antibodies indicated. Tubulin, heat shock protein 90 (HSP90), epidermal growth factor receptor (EGFR) and Alk phos were used as loading controls for cell lysates, cytosol, membrane fractions and conditioned media respectively. A representative immunoblot is seen in A, and schematic in B depicts the position and extent of the deletions in the extracellular domain (ECD) and transmembrane domain (TM). The pooled NTF, CTF1 and CTF2 data quantified by densitometry is shown in panel C. mean $\pm$ SD, n=3, *p < 0.05, **p < 0.001.

Figure 3. Further mapping of the ectodomain cleavage site of FLT1. Panel A and B: HEK293 cells were transiently transfected with pcDNA3, FLT1 or various FLT1 deletion constructs together with a vector expressing secreted alkaline phosphatase (alk phos) and various fractions resolved by SDS page and probed with the antibodies indicated. Tubulin, heat shock protein 90 (HSP90), epidermal growth factor receptor (EGFR) and alk phos were used as loading controls for cell lysates, cytosol, membrane fractions and conditioned media respectively. A representative immunoblot is shown in A and pooled NTF, CTF1 and CTF2 data quantified by densitometry is shown in panel B. mean $\pm$ SD, n=5, *p<0.05, **p<0.001. Panel C: Alignment of the TMD of FLT1 from various species. The TMD is shown between the vertical dotted lines. Residues that are different from human are shown underlined and bolded.

Figure 4.

Quantitation of cFlt1, surface expression and dimerization of Flt1 mutant proteins and cleavage of Flt1-KDR hybrid proteins. Panel A: Free VEGF measured by ELISA in conditioned media (CM) from HEK293 cells transiently transfected with pcDNA3, FLT1 or various FLT1 deletion constructs. The cleaved Flt1 ectodomain binds to secreted VEGF and the reduction in free VEGF in CM reflects the abundance of the cleaved ectodomain. Compared to wild type Flt1, the FLT1 deletion constructs 699-763, 754-763 and 759-763 have markedly diminished ability to reduce free VEGF. Panel B: Expression of Flt1 and various mutant Flt1 proteins were assessed in total lysates and surface fractions of transfected HEK293 cells. Lysates and affinity purified surface proteins were probed with Flag antibody. Endogenous tubulin and EGFR were used as loading controls. Panel C: COS7 cells transiently transfected with pcDNA3, FLT1 or 754-763 was subject to crosslinking with DSS or exposed to vehicle and then cell lysates probed with FLAG or EGFR as a loading control. A shift in Flt1 and 754-763 from the monomeric to the dimeric form is seen with DSS in the FLAG immunoblot and a corresponding shift in size is seen for the EGFR bands. Panel D: HEK293 cells transiently transfected with pcDNA3, KDR, or KDR-Flt1 hybrids and the cleaved HA tagged ectodomain probed by immunoblotting in conditioned media and tGFP-tagged KDR and KDR-Flt1 hybrid proteins probed in total cell lysates. Tubulin was used as a loading control.

Figure 5.

Effect of presenilin 1 and a putative intracellular cleavage site on the generation of Flt1 CTF2. Panel A and B: HEK293 cells were transiently transfected with pcDNA3 or Flt1 with or without a vector expressing presenilin 1 and conditioned media and cellular fractions resolved by SDS page and probed with the antibodies indicated. HA antibody was used with conditioned media and the Flag antibody with cellular fractions. A representative immunoblot is shown in A and pooled NTF, CTF1 and CTF2 data quantified by densitometry is shown in panel B. Panel C: HEK293 cells were transiently transfected with pcDNA3 or myc-tagged Notch1 with or without a vector expressing presenilin 1 and cell lysates resolved by SDS page and probed with the antibodies indicated. NS is a non-specific band seen in all lanes. The 75 kDa Notch 1 isoform that uses an internal start site is labelled Notch 1 and the Notch1 intracellular domain (NICD) is also shown. Panel D and E: HEK293 cells were transiently transfected with pcDNA3, Flt1 or Flt1 V767A and conditioned media and cellular fractions resolved by SDS page and probed with the antibodies indicated. HA antibody was used with conditioned media and the Flag antibody with cellular fractions. A representative immunoblot is shown in D and pooled NTF, CTF1 and CTF2 data quantified by densitometry is shown in panel E.

Figure 6. Effect of Flt1 and various Flt1 mutant constructs on MAP kinase activation. Panel A and B: HEK293 cells transfected with pcDNA3 or various Flt1 constructs and then cell lysates resolved by SDS-PAGE and probed with pp44/42 as well as total p44/42 antibody. A representative immunoblot for pp44/42 in seen in A and pooled data quantified by densitometry is shown in panel C. n=4, mean $\pm$ SD, *p< 0.05. Panel B is representative immunoblot for total p44/42.