The Eph Tyrosine Kinase Receptors EphB2 and EphA2 Are Novel Proteolytic Substrates of Tissue Factor/Coagulation Factor VIIa*

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**Background:** The tissue factor/coagulation factor VIIa (TF/FVIIa) complex cleaves protease-activated receptor 2 (PAR2), but other non-coagulant substrates are not known.

**Results:** Truncated isoforms of the tyrosine kinase receptors EphB2 and EphA2 were formed after FVIIa stimulation.

**Conclusion:** EphB2 and EphA2 are identified as novel proteolytic substrates of TF/FVIIa.

**Significance:** The results provide new insights into PAR2-independent functions of TF/FVIIa.

Tissue factor (TF) binds the serine protease factor VIIa (FVIIa) to form a proteolytically active complex that can trigger coagulation or activate cell signaling. Here we addressed the involvement of tyrosine kinase receptors (RTKs) in TF/FVIIa signaling by antibody array analysis and subsequently found that EphB2 and EphA2 of the Eph RTK family were cleaved in their ectodomains by TF/FVIIa. We used N-terminal Edman sequencing and LC-MS/MS analysis to characterize the cleaved Eph isoforms and identified a key arginine residue at the cleavage site, in agreement with the tryptic serine protease activity of FVIIa. Protease-activated receptor 2 (PAR2) signaling and downstream coagulation activity was non-essential in this context, in further support of a direct cleavage by TF/FVIIa. EphB2 was cleaved by FVIIa concentrations in the subnanomolar range in a number of TF expressing cell types, indicating that the active cellular pool of TF was involved. FVIIa caused potentiation of cell repulsion by the EphB2 ligand ephrin-B1, demonstrating a novel proteolytical event to control Eph-mediated cell segregation. These results define Eph RTKs as novel proteolytical targets of TF/FVIIa and provide new insights into how TF/FVIIa regulates cellular functions independently of PAR2.

The Eph tyrosine kinase receptors (RTKs) constitute 14 members and is the largest RTK family in the human genome. A unique feature of Eph signaling is that the ligands, ephrins, are membrane bound and that signaling preferentially occurs at cell-cell contacts. The Eph-ephrin interaction generates a bidirectional signal both into the Eph-expressing cell through receptor autophosphorylation as well into the ephrin-expressing cell. Ephs and ephrins are divided into A and B classes on the basis of receptor-ligand affinities, where five EphB receptors (EphB1–4, EphB6) bind three B-class transmembrane ephrins (ephrin-B1–3), whereas nine EphA receptors (EphA1–8, EphA10) bind five GPI-anchored ephrin-A ligands (ephrin-A1–5). Interactions between Ephs and ephrins typically occur in large multimeric clusters assembled at the cell surface, where the relative abundances of receptors and ligands determine the outcome of the interaction (1). The Eph-ephrin system thus acts as a global cell positioning system to control intercellular interactions and tissue homeostasis, and play important roles in embryonic development (2–4) and in the organization of adult tissues (5). Upon interactions between Ephs and ephrins, intracellular high affinity molecular tethers are formed that eventually need to be broken to convert adhesion into repulsion and permit signal termination and cell detachment. Accumulating evidence suggest an important role for extracellular proteases, where cleavages in the ectodomain of either the Eph receptor or the ephrin ligand, as demonstrated for EphB2 (6), EphA2 (7), or ephrin-A5 (8), is a requirement for cell segregation.

Tissue factor (TF) is the receptor for coagulation factor VII (FVII/FVIIa), a circulating serine protease, and functions as the physiological initiator of blood coagulation. TF is mostly expressed in epithelial tissues and around blood vessels as a hemostatic envelope (9), but non-physiological TF expression is frequently found in tumor cells (10) and atherosclerotic plaques (11). The proteolytic TF/FVIIa complex has gained increasing attention as an important regulator of cell behavior through activation of cell signaling. TF ligates cell-surface integrins (12), and activates a proinflammatory and proangiogenic response through cleavage of protease-activated receptor 2 (PAR2) (13). TF is closely connected to mechanisms that control cell motility, and TF/FVIIa-PAR2 signaling synergizes with PDGF-BB to promote chemotaxis (14) and angiogenesis (15), and these processes are controlled by the TF cytoplasmic domain. In addition, mouse models have demonstrated an in vivo role for TF signaling in tumor development (12) and obe-

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§¶ The abbreviations used are: RTK, receptor tyrosine kinase; TF, tissue factor; FVIIa, coagulation factor VIIa; PAR2, protease-activated receptor 2; MMP, matrix metalloproteinase; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; PLA, proximity ligation assay.
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sity (16). Although the link between TF/FVIIa and PAR2 appears to be close, it has become clear that PAR2-independent signaling also exists as the antiapoptotic effect of FVIIa is independent of both PAR1 and PAR2 (17–18).

In this article we addressed the involvement of RTKs in TF/FVIIa signaling, and guided by antibody-based RTK signaling arrays we identified Eph receptors as new targets of TF/FVIIa proteolytic activity. We report that EphB2 and EphA2 were cleaved near the N terminus in response to FVIIa, and the cleavage site was identified by N-terminal Edman sequencing and LC-MS/MS analysis to occur after a conserved arginine residue in the ligand-binding domain. FVIIa affected the EphB2-ephrin-B1 interaction and led to increased cell repulsion. Our results suggest a novel proteolytic mechanism for TF/FVIIa to control cellular interactions and tissue organization following coagulation activation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were used: N-terminal EphB2 (AF467) and EphB4 (AF3038) were from R&D Systems. C-terminal EphB2 (37–1700) was from Invitrogen. EphB3 (M01, clone 1B3) was from Abnova Corporation. EphA2 (6997), GAPDH (2118), ERK (9107), and phospho-ERK (4370) were from Cell Signaling Technology. Mouse monoclonal anti-TF antibodies used for blocking experiments (clones 10H10, 5G9, and 5B7) were kind gifts from Professor James Morrissey (University of Illinois). The rabbit polyclonal PAR2-blocking antibody was a kind gift from Professor Wolfram Ruf (Scripps Institute). Recombinant FVIIa and FFR-FVII were kind gifts from Professor L. C. Petersen (Nordovist AS). The PAR1 and PAR2 agonist peptides SFLLRN and SLIGKV were from Sigma. Human α-thrombin and human FX were from Enzyme Research Laboratories. Recombinant ephrin-B1 Fc chimeras were from R&D Systems. Control Fc fragment and anti-Fc IgG were from Jackson ImmunoResearch. MG132, bafilomycin A1, GM6001, and TAPI-1 were form 

RTK Antibody Arrays—RTK signaling was screened using antibody arrays (Cell Signaling Technology) according to the manufacturer’s instructions. Briefly, MDA-MB-231 cells and primary human fibroblasts were stimulated as indicated in Table 1 for 3 and 30 min (MDA-MB-231) or 30 min (fibroblasts) and lysed in non-denaturing cell lysis buffer. Cell lysates were added to array slides with target capture antibodies spotted in duplicate and incubated overnight at 4 °C. To detect tyrosine phosphorylation of the bound RTKs, a detection antibody mixture containing a biotin-linked pan-phosphotyrosine antibody and DyLight 680-linked streptavidin was added. After washing and drying, images of the array slides were captured with the Odyssey System (Licor Biosciences) and spots corresponding to the different RTKs were identified by their coordinates and quantified with the Odyssey Imaging Software version 3.0.

Gel Electrophoresis and Western Blot—Gel electrophoresis and Western blot were performed using NuPage BisTris gels (Invitrogen) and the Odyssey Infrared Imaging System (Licor Biosciences) as described previously (18). For non-reduced samples, reducing agent was omitted from the SDS-PAGE sample buffer.

Deglycosylation of Total Cell Lysates—Total cell lysates from MDA-MB-231 cells were subjected to deglycosylation by N-glycosidase F (Roche Applied Science). Briefly, 0.4% SDS was added to 10 μg of protein, and samples were boiled to denature proteins. 2 Units of N-glycosidase F and 0.5% Nonidet P-40 were added and samples were incubated for 6 h at 37 °C and analyzed by Western blot.

In Situ Proximity Ligation Assay—The in situ PLA assay was performed according to the manufacturer’s instructions, using reagents supplied by the manufacturer (Olink Bioscience). In brief, MDA-MB-231 cells and U251 cells were cultured on chamber slides, fixed in 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Slides were then blocked and incubated with primary antibodies toward TF (10H10, 1:200) and EphB2 (N terminus, 1:200). TF and EphB2 antibodies in close proximity were visualized with secondary antibodies connected to oligos that served as templates for rolling circle amplification. The rolling circle amplification products were hybridized to fluorescently labeled probes and cells were then counterstained with DAPI and images captured at ×40 with a fluorescence microscope. The PLA signals were quantified using the Duolink image tool (Olink Bioscience).

N-terminal Edman Sequencing—EphA2 was purified by immunoprecipitation from FVIIa-stimulated MDA-MB-231 cells and subjected to SDS-PAGE and Western blot. Membranes were stained with Coomassie Brilliant Blue, and bands corresponding to cleaved EphA2 were excised and submitted to N-terminal sequencing, performed as fee-for-service by Alphalyse on an ABI Procie 494 sequencer (Applied Biosystems) as described on their website.

MS Analysis of EphA2—EphA2 was purified from FVIIa-stimulated MDA-MB-231 cells as described above and subjected to SDS-PAGE. The gel was then stained with Coomassie Brilliant Blue, and gel bands corresponding to cleaved or full-length EphA2 were reduced, alkylated, and enzymatically digested as described elsewhere (19). The proteins were digested either by trypsin (Roche Applied Science) or chymotrypsin (Wako Chemicals USA) at 12.5 ng/μl overnight. The peptides were extracted by sonication in 60% acetonitrile and 5% formic acid. Finally, the samples were dried in a SpeedVac system (Eppendorf, Concentrator Plus) and resolved in 0.1% formic acid. The enzymatic peptides were separated in reversed phase on a 10-cm long C18-A2 column, inner diameter 75 μm (Thermo Scientific), using mobile phase A = 0.1% formic acid.
TABLE 1
Eph receptor activity after FVIIa and PAR2-AP stimulation and Eph receptor tyrosine kinases are involved in TF/FVIIa signaling

|               | MDA-MB-231 | Human fibroblasts |
|---------------|------------|-------------------|
|               | Ctrl       | FVIIa  | PAR2-AP  | Ctrl       | FVIIa  | PAR2-AP  |
| EphB1         | 100        | 53.2 ± 1.9 | 124.2 ± 23.7 | 100        | 54.9 ± 1.5 | 106.2 ± 25.1 |
| EphB3         | 100        | 66.5 ± 3.0 | 109.1 ± 9.3  | 100        | 62.8 ± 0.4 | 93.3 ± 13.3  |
| EphB4         | 100        | 83.2 ± 9.6 | 91.2 ± 12.5  | 100        | 77.8 ± 31.4 | 100.0 ± 47.1 |
| EphA1         | 100        | 91.5 ± 8.6 | 84.2 ± 18.8  | 100        | 88.1 ± 7.7 | 72.4 ± 16.3  |
| EphA2         | 100        | 74.2 ± 1.2 | 110.8 ± 3.5  | 100        | 60.0 ± 14.1 | 83.3 ± 23.6  |
| EphA3         | 100        | 102.8 ± 1.9 | 96.6 ± 6.8   | 100        | 87.2 ± 0.8 | 91.0 ± 9.3   |

and B = 0.1% formic acid, 99.9% acetonitrile. The gradient used was 4–50% B in 60 min. The peptides were electrosprayed online to an LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Scientific). Tandem mass spectrometry was performed applying collision-induced dissociation. Proteome Discoverer 1.4 (Thermo Scientific) was applied to compare detected peptides to in silico digested protein sequences of EphA2 obtained from Swiss-Prot. For tryptic and chymotryptic peptides, 2 and 6 missed cleavage sites were allowed, respectively.

Transwell Cell Migration Assay—Fluoroblok Transwell inserts in 24-well format with 8-μm pore size were purchased from BD Biosciences. Both sides of the inserts were coated with 10 μg/ml of collagen IV in PBS at 4 °C overnight. MDA-MB-231 cells (50,000 per well) in basal RPMI medium supplemented with 0.1% BSA were added to the inserts and cells were allowed to migrate for 5 h at 37 °C. The inserts were then fixed in 4% paraformaldehyde and stained with 5 μg/ml of Hoechst 33342 (Merck Millipore) to visualize cell nuclei. Images of the underside of the inserts from 3 randomly selected fields per well were generated using an Axiovert 40 CFL inverted fluorescence microscope using a ×20/0.30 objective and Axiovision 4.8 software (Carl Zeiss). Cells that had migrated through the membrane were counted with the Cell Profiler image analysis software, with settings that excluded cells within pores.

siRNA Knockdown of EphB2—MDA-MB-231 cells were transfected with scrambled or EphB2 siRNA (catalog number S102224789, Qiagen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Cells were re-transfected after 48 h and subjected to the Transwell cell migration assay 72 h after the initial transfection. A fraction of the cells were lysed and analyzed for EphB2 protein expression by Western blot.

Statistics—Results were tested for statistical significance by the two-tailed unpaired Student’s t test. p values equal to or below 0.05 were considered statistically significant. Results are expressed as means with error bars indicating the S.D.

RESULTS

Eph Receptor Tyrosine Kinases Are Involved in TF/FVIIa Signaling—To generate a global view of RTK activity downstream of TF/FVIIa signaling we used antibody-based array analysis to examine expression of phosphorylated RTKs in MDA-MB-231 cells and primary human fibroblasts. In both cell types, we observed that FVIIa stimulation reduced the phosphotyrosine signal compared with untreated cells for members of the EphB RTK family, with the largest effects seen for EphB1 (53.2 ± 1.9% of control in MDA cells, 54.9 ± 1.5% in fibroblasts) and EphB3 (66.5 ± 3.0% in MDA cells, 62.8 ± 0.4% in fibroblasts), whereas the EphB4 signal was mildly decreased (83.2 ± 9.6 in MDA cells, 77.8 ± 31.4% in fibroblasts), implying a change in Eph RTK activity induced by FVIIa (Table 1). Of the three receptors of the EphA subfamily present on the array, the EphA2 signal was similarly reduced by FVIIa (74.2 ± 1.2% in MDA cells, 60.0 ± 14.1% in fibroblasts), whereas the EphA1 and EphA3 values were either unaffected or slightly reduced by FVIIa. Stimulation with a PAR2-agonist peptide (PAR2-AP) did not result in any consistent changes in the Eph phosphotyrosine signal except for a slight reduction in EphA1 values, indicating that FVIIa alters Eph activity independently of PAR2.

The array results prompted us to further investigate how TF/FVIIa might regulate the function and expression of Eph receptors, and we used MDA-MB-231 cells to study expression levels of a selected number of Eph receptors based on the antibody array screening. Cells were stimulated with FVIIa for different time points, and expression levels of EphB2, EphB3, EphB4, and EphA2 were analyzed by Western blot. Unfortunately EphB1 had to be excluded from further analysis because we could not find a specific antibody. Instead, the other EphB family member EphB2 was included for further studies. Western blotting experiments revealed that stimulation with 10 nM FVIIa markedly reduced the expression of full-length EphB2 and lead to the rapid appearance of a reduced molecular mass isoform of EphB2, migrating around 110 kDa compared with 125 kDa for full-length EphB2 (Fig. 1A). A short EphA2 isoform of similar size as short EphB2 was readily detected after 1 and 6 h, whereas a weak EphB4 band of reduced size was seen on high exposure blots after 6 h but not 1 h. No additional EphB3 isoforms were detected after FVIIa stimulation.

Time and concentration curves revealed that the short EphB2 isoform was formed within 10 min of exposure to 10 nM FVIIa, and was detectable after 6 h treatment with a subnanomolar concentration of FVIIa (Fig. 1B). The short EphA2 isoform was evident on high exposure Western blots after 30 min of FVIIa stimulation, but concentration curves revealed that higher concentrations (5 nM) were needed than for EphB2, and only a fraction of the total receptor population migrated at the reduced molecular weight even after prolonged FVIIa stimulation.

These results indicated that FVIIa was very potent in generating the short EphB2 isoform, but because MDA-MB-231 cells
express very high levels of TF we also sought to verify these results in cells with physiological TF levels. We could detect the short EphB2 isoform following FVIIa stimulation in both primary human fibroblasts (Fig. 1C) and U251 glioblastoma cells (Fig. 1D), both of which express roughly 10 times less TF compared with MDA-MB-231 cells as measured by flow cytometry (data not shown). 0.5 nM FVIIa produced detectable levels of the short isoform in both cell types, and saturation seemed to be reached around 1 nM FVIIa.

The Short EphB2 Isoform Is Cleaved in the Extracellular Domain—We next performed a series of experiments in MDA-MB-231 cells to further characterize the short EphB2 isoform induced by FVIIa. We observed that it was insensitive to the protein synthesis inhibitor cycloheximide or inhibitors of intracellular degradation, and Western blots of deglycosylated samples showed that both full-length and short EphB2 exhibited a shift in gel motility (Fig. 2, A–C). These results indicated a change in the primary structure of EphB2 and a proteolytical cleavage at the cell surface as the mechanism behind the gel shift. We next aimed to detect the cleaved EphB2 fragment in cell culture supernatants, and because the EphB2 antibody was of polyclonal origin and directed toward the entire extracellular domain a fraction of the antiseraum could possibly react with the released peptide. Indeed, Western blot on supernatants from MDA-MB-231 cells revealed that the antibody reacted with a fragment migrating at 15 kDa in the supernatant from FVIIa-treated cells, which corresponded well in size with the reduction in molecular weight of the short isoform and was absent in control supernatants (Fig. 2D). An EphB2 antibody toward an epitope in the intracellular domain detected both cell-bound isoforms, but did not react with the N-terminal fragment in the supernatant. Taken together, these results demonstrate that EphB2 is cleaved near the N terminus in response to FVIIa.

EphB2 Is Cleaved by TF/FVIIa—We continued by investigating the mechanisms by which FVIIa causes EphB2 cleavage, and to verify the specificity and TF dependence of our findings we used three previously characterized clones of TF-blocking antibodies (20, 21) and active site inhibited FVII (FFR-FVII).
Although mAbs 5G9 and 5B7 blocked the effects of FVIIa (Fig. 3A), mAb10H10, which selectively blocks PAR2 activation by TF/FVIIa (22), did not prevent EphB2 cleavage. Furthermore, blocking experiments with FFR-FVII corroborated a specific mechanism and demonstrated the requirement for FVIIa proteolytic activity (Fig. 3B). The prototypic TF/FVIIa signaling mechanism is PAR2 activation, but in agreement with the inability of mAb10H10 to interfere with EphB2 cleavage, experiments with a blocking antibody that binds to the cleavage site in PAR2 demonstrated that PAR2 is not mediating this effect of FVIIa (Fig. 3C). We also verified our mAb10H10 batch and found that it effectively prevented FVIIa-induced IL8 mRNA as previously reported (data not shown).

We instead reasoned that either EphB2 was cleaved directly by TF/FVIIa or by downstream coagulation proteases generated by TF/FVIIa. However, neither the ternary TF/FVIIa/FXa complex, active thrombin, nor agonists to the PAR1/2 receptors that coagulation proteases act upon induced EphB2 cleavage (Fig. 3, D and E), demonstrating that EphB2 cleavage is uncoupled from TF/FVIIa procoagulant activity.

Last, although no such mechanism is known, we also considered it a theoretical possibility that FVIIa activated a second unknown protease, which in turn cleaved EphB2. We screened through a set of broad-spectrum protease inhibitors and solely leupeptin, an inhibitor of serine and cysteine proteases and to a lesser extent aprotinin, a serine protease inhibitor reported to have low activity toward FVIIa (23), reduced EphB2 cleavage (Fig. 3F). Concentration curves with leupeptin showed that FVIIa-induced ERK phosphorylation, known to occur through PAR2 cleavage (12) was dose dependently inhibited by leupeptin at the same concentrations as EphB2 cleavage suggesting that leupeptin acted directly on FVIIa (Fig. 3F). In further support of a direct cleavage we found that TF and EphB2 co-localized constitutively in MDA-MB-231 cells by the in situ proximity ligation method (24) (Fig. 3G). The number of detectable TF-EphB2 complexes in U251 cells was considerably lower, in agreement with a reduced level of EphB2 cleavage and a slower onset. Altogether, these results clearly indicate that cleavage occurs directly by TF/FVIIa.

Identification of the Cleavage Site in EphA2 and EphB2—To identify the cleavage site we used N-terminal Edman sequencing and LC-MS/MS analysis, and because MDA-MB-231 cells express moderate levels of EphB2 but high levels of EphA2, the short EphA2 isoform was used for these analyses. N-terminal sequencing of cleaved EphA2 determined the amino acid sequence of residues 2–5 to be VKLN, whereas the most N-ter-
terminal residue could not be determined and Ser, Gly, His, Arg, or Met were compatible with the sequencing data. Of these possible amino acid sequences, 160HVKLN165 mapped to the ligand-binding domain of EphA2 and was preceded by an arginine residue, indicating a cleavage site at the Arg159-His160 bond (Fig. 4A). The results were verified by LC-MS/MS analysis, which confirmed that cleaved EphA2 was N terminally truncated. Upon analysis of the chymotryptic digest of cleaved EphA2 two peptides containing the non-chymotryptic 160HVKLN165 end were detected (HVKLNVEERSVGPL and HVKLNVEERSVGPLTRKGF). These peptides were evidence of a unique N terminus and confirmed the N terminus determined by Edman sequencing. In addition, LC-MS/MS analysis revealed the presence of at least one additional truncated EphA2 variant as peptides mapping N terminally to the Arg159-His160 cleavage site were detected. The most N-terminal detected peptides were a chymotryptic peptide starting at tyrosine 123 and a tryptic peptide starting at leucine 138. Although
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we failed to identify the exact N terminus of this EphA2 isoform; our results suggest it to be located within the 120NLY122 triplet based on peptides detected for full-length but not truncated EphA2, as analysis of full-length EphA2 generated an abundance of peptides mapping N terminally to the truncated isoforms described above (data not shown).

Based on the tryptic serine protease specificity of FVIIa with a requirement for an arginine residue at the P1 position (25) we concluded that cleavage by TF/FVIIa takes place at the Arg155-His156 bond in EphA2. We performed sequence alignments of the cleavage area, which revealed that the key EphA2-Arg159 residue was conserved in EphB2 but not in EphB3 or EphB4 (Fig. 4B), which were not significantly cleaved by TF/FVIIa. Furthermore, a cross-species alignment showed that this arginine was perfectly conserved throughout evolution (Fig. 4C).

We calculated the predicted molecular weights of EphB2 and EphA2 cleaved at this position using the ExPaSy Compute pl/MW tool, which corresponds with the observed gel migration patterns. Full-length EphB2 and EphB2 cleaved after arginine 155 were predicted to be 108 and 92 kDa, respectively, but due to glycosylations EphB2 migrated with a higher molecular mass than predicted from the sequence. However, the gel migration pattern of deglycosylated EphB2 (Fig. 2C) was in agreement with what was expected both for the full-length and cleaved isoforms. The released N-terminal fragment of EphB2 was predicted to be 15.8 kDa, in perfect agreement with its migration pattern on-gel just above the 15-kDa marker, supporting that the cleavage site in EphB2 is located at the Arg155-Val156 bond.

Formation of Truncated EphA2 and EphB2 Is Regulated by the Redox Status of a Disulfide Bond—Upon further examination of the Eph ligand-binding domain we found a conserved disulfide bridge (Cys70-Cys188 in EphA2, Cys92-Cys184 in EphB2) that connected two cysteine residues located upstream and downstream, respectively, of the cleavage site (Fig. 5A).

SDS-PAGE performed during non-reducing conditions to preserve disulfide bridges revealed that EphA2 and EphB2 in FVIIa-treated cells migrated similarly on gels as EphA2 and EphB2 in untreated cells during these conditions, suggesting that the cell-bound isoforms were kept associated with the N-terminal fragment by the disulfide bond (Fig. 5B). In addition to being detected in the cell culture supernatant, the 15-kDa N-terminal fragment of EphB2 was detected by the extracellular EphB2 antibody in reduced cell lysates (Fig. 5B), and correlated with the extent of EphB2 cleavage. This band disappeared in non-reduced samples, demonstrating that it corresponds to the N-terminal part of EphB2, which apart from being released to the cell medium remained cell-associated through the disulfide bond.

FVIIa Potentiates EphB2-dependent Repulsion by Ephrin-B1 in a Transwell Cell Migration Assay—To determine the functional implications of our results we subjected MDA-MB-231 cells to a Transwell cell migration assay and evaluated the cellular response to the EphB2 ligand ephrin-B1 in the presence or absence of FVIIa. 5% FBS was used as a chemoattractant and in agreement with previous studies, ephrin-B1 induced repulsion and reduced the number of migrating cells. 10 nM FVIIa significantly potentiated inhibition of cell migration by ephrin-B1, both at medium (2 µg/ml) and high (4 µg/ml) concentrations (Fig. 6A). Addition of FVIIa did not alter the migration toward 5% FBS alone. To verify that FVIIa increased ephrin-B1 repulsion through the EphB2 receptor, we silenced its expression by siRNA. EphB2-silenced cells displayed reduced basal levels of migration in agreement with previous studies (26), but still responded to ephrin-B1, which may act through EphB3 also expressed by these cells (27). However, the migration toward ephrin-B1 was unaffected by FVIIa upon EphB2 knockdown, demonstrating that FVIIa impacts the EphB2-ephrin-B1 interaction by increasing cell repulsion (Fig. 6B).

DISCUSSION

In the present study, we report on the identification of novel truncated variants of Eph receptors EphB2 and EphA2, identified by antibody array analysis and formed as a result of proteolytical cleavages within the ligand-binding domain by TF/FVIIa.

The antibody array screening was used as an unbiased approach to investigate RTK involvement in TF/FVIIa signaling with the most consistent finding being the reduction in Eph phosphotyrosine signal after FVIIa stimulation, which we interpreted as a change in Eph activity induced by FVIIa. The array was run with native samples and has been noted previously, a decrease in signal in this context can be due either to a direct decrease in tyrosine phosphorylation or the formation of multiprotein complexes effectively masking the target epitope (28, 29). In any case the array served to identify Eph RTKs as targets of TF/FVIIa signaling, which led us to identify a direct cleavage by TF/FVIIa in a subset of Eph RTKs.

FIGURE 3. EphB2 is cleaved by TF/FVIIa. A and B, EphB2 cleavage by FVIIa is dependent on TF and the proteolytical activity of FVIIa. MDA-MB-231 cells were pre-treated for 30 min with 50 µg/ml of TF-blocking antibodies (A) or 100 nM active site-inhibited FVII (FFR-FVII) (B) before 10 nM FVIIa was added for 6 h. C–E, EphB2 cleavage by FVIIa is independent of PAR2 and downstream coagulation proteases. MDA-MB-231 cells were pre-treated for 30 min with 100 µg/ml of PAR2-blocking antibody before 10 nM FVIIa was added for 6 h (C), or treated for 6 h with the indicated agonists (PAR agonist peptides 50 µM, thrombin 10 nM) (D and E). F and G, EphB2 is cleaved by TF/FVIIa. F, upper panel: MDA-MB-231 cells were pre-treated with either DMSO or the indicated inhibitors for 30 min before 10 nM FVIIa was added for 1 h. The following inhibitors were used, with targets and working concentrations indicated in parentheses: leupeptin (serine and cysteine proteases; 50 µM), aprotonin (serine proteases 50 µM), TAPI-1 (ADAMs, 10 µM), GM6001 (MMPs, 25 µM), E64 (cysteine proteases, 50 µM), and pepstatin (aspartic proteases, 50 µM). Samples were analyzed for EphB2 expression by Western blot. n = 3–4 representative gels are shown. Lower panel, MDA-MB-231 cells were pre-treated with increasing concentrations of leupeptin as indicated for 30 min, and 10 nM FVIIa was added for 1 h and EphB2 expression and ERK phosphorylation analyzed by Western blot. n = 3 representative gels are shown. G, TF and EphB2 co-localize constitutively in MDA-MB-231 cells. The Duolink in situ proximity ligation assay (in situ PLA) was used to detect TF-EphB2 complexes in MDA-MB-231 and U251 cells. Cells were incubated with antibodies toward TF and EphB2, and bound antibodies in close proximity representing co-localizing TF-EphB2 were visualized using DNA oligo-connected secondary antibodies and rolling circle amplification as described under “Experimental Procedures.” Each detected TF-EphB2 antibody pair generated an rolling circle amplification product, which was subsequently hybridized to fluorescently labeled probes and is visible as red signal. Blue signal represents DAPI-stained DNA. The average number of detected TF-EphB2 complexes per cell was 39 ± 28.3 for MDA-MB-231 cells and 6.2 ± 2.9 for U251 cells. n = 3 (MDA-MB-231) or 2 (U251) representative images are shown. DMSO, dimethyl sulfoxide.
We subsequently used different experimental approaches to conclude that EphB2 and EphA2 are cleaved directly by TF/FVIIa and define Eph receptors as novel substrates of the proteolytical TF/FVIIa complex. The cleavage was mapped to occur after a key arginine residue in the ligand-binding domain in agreement with the tryptic serine protease activity of FVIIa.
and with what is observed in the known FVIIa substrates PAR2, FX, and FIX (30–32). Antibody blocking experiments and agoni- 

data verified that cleavage occurs independently of both the 

prototypic TF/FVIIa receptor PAR2 and downstream coagula-


tion proteases, the known mechanisms of TF/FVIIa signaling. 

EphB2 was cleaved by subnanomolar concentrations of FVIIa 

in all cell lines but not by the TF/FVIIa/FXα complex, suggest-

ing that cleavage occurs by FVIIa bound to the active cellular 

pool of TF with a high affinity for FVIIa, yet by a pathway that 

is uncoupled from FX activation. Cleavage was only inhibited 

by high concentrations of serine protease inhibitors, consistent 

with the notion that these inhibitors acted directly on FVIIa, 

although at this point we cannot completely rule out that the 

cleavage reaction involves a second serine protease activated 

or induced by TF/FVIIa. Importantly, TF and EphB2 co-localized 

constitutively in MDA-MB-231 cells in agreement with the 

extensive EphB2 cleavage we observed after prolonged FVIIa 

stimulation.

The cleavage site was mapped to the J-K loop of the EphA2/ 

EphB2 ligand-binding domain, a region readily accessible to a 

protease as demonstrated by the crystal structures, which is 

a prerequisite for a cleavage reaction. Moreover, it is located in 

a part of the ligand-binding domain involved in high affinity 

interactions with ephrin ligands (33), further supporting that 

the cleavage site indeed is accessible for interactions with other 

proteins. Interestingly, the arginine residue at the cleavage site 

plays an important role in ephrin binding and forms a salt 

bridge with an aspartate residue in the ephrin ligand, similar to 

the FVIIa cleavage reaction where the acidic aspartate residue 

in the active site forms a salt bridge with the basic arginine 

residue in the scissile bond to be cleaved (25).

Apart from verifying the N terminus determined by Edman 

sequencing, LC-MS/MS analysis detected at least one addi-

tional truncated EphA2 isoform, with an N terminus predicted 

to be located around 40 amino acids upstream of the major 

cleavage site. As the predicted N terminus of this isoform did 

not contain any arginine residues, cleavage was not compatible 

with cleavage by a tryptic serine protease like FVIIa.

In further support of the relevance of such a mechanism, disul-

fide bond and reduction of a disulfide, are needed to generate the free 

ligand binding to allow cells to detach, which was reflected in 

the cell migration assay as increased repulsion. Our results indi-

cate that two sequential events, including a proteolytic cleavage 

and reduction of a disulfide, are needed to generate the free 

form of the N-terminal EphB2 fragment. Interestingly, a similar 

mechanism has been observed previously in the case of 

angiostatin, which is a peptide released from plasminogen upon 

the reduction of two disulfides and a proteolytic cleavage (37).

The TF/FVIIa cleavage site in EphB2 was spanned by a disul-

fide bridge, which needs to be reduced for the N-terminal frag-

ment to be released. Because the disulfide bond appeared to be 

intact in a major fraction of EphB2 receptors in our cell line 

model systems, this might explain why cells still responded to 

ephrin-B1. A possibility is that the disulfide bond is broken after 

ligand binding to allow cells to detach, which was reflected in 

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In further support of the relevance of such a mechanism, disul-

FIGURE 4. Identification of the cleavage site in EphA2 and EphB2. A, upper panel: purification of cleaved EphA2. MDA-MB-231 cells were treated with 25 nM 

FVIIa overnight and EphA2 was immunoprecipitated (IP), subjected to SDS-PAGE, and the gel stained with Coomassie Brilliant Blue. Arrows indicate the bands 

corresponding to full-length and truncated EphA2, which were cut out for LC-MS/MS analysis. Lower panel: identification of the EphA2 cleavage site. Cleaved 

EphA2 was subjected to N-terminal Edman sequencing, and cleaved and full-length EphA2 were digested with chymotrypsin or trypsin and subjected to 

LC-MS/MS analysis as described under “Experimental Procedures.” The figure displays the amino acid sequence of the EphA2 ligand-binding domain. Arrow- 

heads indicate the predicted cleavage sites for chymotrypsin and trypsin, respectively. The N terminus detected by Edman sequencing is indicated in bold.

Peptides detected by LC-MS/MS analysis of chymotrypsin or trypsin-digested cleaved EphA2 are indicated as lines, with the additional sequence coverage 

N terminally from the Edman-verified sequence indicated in green bold. In chymotrypsin-digested cleaved EphA2, the two peptides, HVLKLNVEERSVGPLTR 

and HVLKLNVEERSVGPLTRKGF, containing the non-chymotryptic end HVLKLN165 were detected (dashed lines). The most N-terminal peptides detected for cleaved 

EphA2 were YAESDLDYGTFNQRKFL (chymotrypsin digestion) and LFTKIDTDIAPDEITVSSDFEAR (trypsin digestion). B, sequence alignment and three-dimen-

sional structure of the cleavage region. The key C-terminal arginine residue identified in EphA2 and EphB2 was not conserved in EphB4, and the EphB4 sequence 

contained other closely located arginine residues that could possibly provide a basis for TF/FVIIa cleavage sites. However, apart from the amino acid sequence, factors such as the 

three-dimensional structure of the protein and accessibility to proteases will determine whether a proteolytic cleavage can occur (34), which may explain the preference of TF/FVIIa for EphB2 and EphA2. 

Proteolytic cleavages have been reported in a number of cell 

adhesion molecules, e.g. E-cadherin (35) and integrins (36), pro-

posed to function as a regulatory mechanism in control of the 

cell adhesion/detachment dichotomy. Eph receptors share 

many features with cell adhesion molecules including the need 

for a mechanism that allows interacting cells to disengage, and 

in accordance with this concept EphB2 cleavage by TF/FVIIa 

resulted in potentiated repulsion by its ligand ephrin-B1. Importantly, we show that potentiation of ephrin-B1 by FVIIa requires EphB2 expression, demonstrating that FVIIa specif-

ically impacts the outcome of the ephrin-B1-EphB2 interaction.

Furthermore, our findings are in line with other reports where 

proteolytic cleavages of Eph receptors are associated with cell 

segregation. Sugiyama et al. (7) found that MT1-MMP-medi-

ated cleavage in EphA2 was associated with increased single cell 

invasion of breast cancer cells, and Lin et al. (6) reported that 

extracellular MMP-dependent cleavages of EphB2 were 

required for ligand-induced repulsion. However, cleavage by 

TF/FVIIa was insensitive to MMP inhibition, demonstrating a 

distinct mechanism. 

The TF/FVIIa cleavage site in EphB2 was spanned by a disul-

fide bridge, which needs to be reduced for the N-terminal frag-

ment to be released. Because the disulfide bond appeared to be 

intact in a major fraction of EphB2 receptors in our cell line 

model systems, this might explain why cells still responded to 

ephrin-B1. A possibility is that the disulfide bond is broken after 

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mechanism has been observed previously in the case of 

angiostatin, which is a peptide released from plasminogen upon 

the reduction of two disulfides and a proteolytic cleavage (37). 

In further support of the relevance of such a mechanism, disul-


**TF/FVIIa Cleaves EphB2 and EphA2**

**EphA2 ligand binding domain**

28  EVVLLDFAAAGGELGWLTHYPYGKGDLMQNM
61  DMPIMYSVCNVWSGDQDNWLPNVRGAEERIFIELKFTVRCNSFPGGASSCKETFN
121  LLLAEEDLLYGTSTQKRFLTTIDTIADEITVSSDFEARRNVKLEERVSYGFLTRKSFYL
181  AFQDIGACVALLSRYVYKKEFELLQ

**EphB2 ligand binding domain**

20  EVTVDFSTTQATAGNLGKPDDVSFGVQGMDTNTIRTYQ
61  VCNVFESSQNLRTKFIIRGAGRHIHVEMKFSVRCSSISPSVFGSCSCEFNLYYYEADF
121  DSAATFNTNNMENFVKRTNVIADASEFSQVDLGGRVMKINTEVSFGFVSFGYLFQD
181  YGGCMLIAVFRFNYKCPRIIQ

**C**

Cleaved

Uncleaved
fide bonds are being recognized as dynamic structures (38), and a recent study found the oxioreductase protein-disulfide isomerase to dynamically control the redox state of EphB2 (39). Protein-disulfide isomerase has also been implicated in regulation of TF activity by acting on the Cys186-Cys209 disulfide bond (22) and we recently found in a two-dimensional DIGE proteomic study that the reductant thioredoxin was significantly up-regulated upon FVIIa binding to TF (40).

**FIGURE 5.** The cleavage site in EphB2 and EphA2 is spanned by a disulfide bridge. A, upper panel: image of the three-dimensional structure of EphA2 (magenta) and EphB2 (gray) with Cys186-Cys209 (EphA2) and Cys184-Cys209 (EphB2) residues indicated in purple and the cleavage site in red. The image was generated as described in the legend to Fig. 4B. Lower panel, sequences of the ligand-binding domains of EphA2 and EphB2 with the Cys186-Cys209 disulfide bonds indicated in bold red and the arginine 159 (EphA2)/arginine 155 (EphB2) residue at the cleavage site in bold.

**FIGURE 6.** FVIIa potentiates EphB2-dependent repulsion by ephrin-B1 in a Transwell cell migration assay. Transwell cell migration assays were performed according to Miao et al. (46) with some modifications. MDA-MB-231 cells were migrating toward 5% FBS supplemented with either 4 μg/ml of Fc control or 2–4 μg/ml of ephrin-B1-Fc, which were pre-clustered 1 h at room temperature with 1:10 anti-Fc IgG prior to experiments. For the FVIIa groups, 10 nM FVIIa was added both to the upper and lower chambers. A, potentiation of ephrin-B1 by FVIIa. FVIIa did not affect the number of migrating cells toward 5% FBS + 4 μg/ml of Fc (100.8 ± 13.5%, p = 0.71), whereas inhibition of cell migration by 2 μg/ml of ephrin-B1 (48.8 ± 13.7 versus 68.2 ± 14%, p = 0.019) and 4 μg/ml of ephrin-B1 (24 ± 6.7 versus 45 ± 13.8%, p = 0.0003) was significantly potentiated by FVIIa.

B, ephrin-B1 potentiation by FVIIa is dependent on EphB2. Cells transfected with siRNA knockdown for EphB2 or scramble (Scr) as control were subjected to the Transwell cell migration assay migrating toward 5% FBS and 4 μg/ml of ephrin-B1. EphB2 knockdown was verified by a Western blot, of which a representative blot is shown. FVIIa potentiated inhibition of cell migration of 4 μg/ml of ephrin-B1 in Scr-transfected cells (49.7 ± 11.5 versus 68.0 ± 14.3%, p = 0.034). EphB2 silencing reduced the number of migrating cells toward 5% FBS + 4 μg/ml of Fc (42.8 ± 7.4%), but abolished the potentiating effect of FVIIa on ephrin-B1 (21.5 ± 4.7 versus 23.2 ± 5.2%, p = 0.58). Results are presented as percentages of control cells migrating toward 5% FBS + 4 μg/ml of Fc. Images show Hoechst-stained nuclei on the underside of the Transwell inserts, corresponding to the number of migrating cells. The experiments were performed in triplicates and repeated two to three times. *, p < 0.05; ***, p < 0.001.
The in vivo relevance of EphB2 cleavage by TF/FVIIa remains to be determined. TF and Eph receptors appear to be co-expressed in disease states with high TF expression, such as solid cancers (13, 3) and on monocytes/macrophages in atherosclerotic plaques (11, 41, 42). We speculate that upon tissue damage when FVIIa can access and bind to TF, EphB2 cleavage by TF/FVIIa and associated ephrin-B1-dependent cell segregation may contribute to plasticity and cell motility in normal as well as pathological conditions. The subnanomolar potency of FVIIa, far below the 10 nM circulating concentration of total FVII in vivo (43) demonstrates that selective events are induced in primary cells at physiological FVIIa concentrations and supports that our findings are of broader relevance. We also found the amino acid sequence in the cleavage region to be extensively conserved throughout evolution, further corroborating a functional importance of our findings.

In conclusion, our study identifies EphB2 and EphA2 as novel substrates for the proteolytic TF/FVIIa complex. It conceptualizes that TF/FVIIa non-coagulant activity can occur independently of PAR2 and adds the coagulation system to the growing number of proteolytic mechanisms that control Eph receptor function.

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