Fibronectin Type I Repeat Is a Nonactivating Ligand for EphA1 and Inhibits ATF3-dependent Angiogenesis

Junko Masuda, Ryosuke Usui, and Yoshiro Maru
From the Department of Pharmacology, Tokyo Women’s Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

ATF3 stimulated promoter activity of EphA1 by 3.4-fold in ATF3-dependent angiogenesis in vitro. Although tyrosine kinase activation of EphA1 was dispensable, binding of EphA1 to fibronectin through its type I repeat played an essential role in the angiogenesis. Recombinant proteins containing fibronectin 10th to 12th type I repeat (I10–12) but not I12 could inhibit the angiogenesis in vitro by competitively targeting EphA1 with the full-length fibronectin. However, I12 acquired a higher affinity toward EphA2 with Ki 18 nM and inhibited vascular endothelial growth factor-dependent angiogenic invasion in a Matrigel plug assay.

The Eph receptor tyrosine kinase family consists of 16 members and is subdivided into group A (EphA) and B (EphB). They have nine ligands called ephrins that are also classified into A (ephrin-A) and B (ephrin-B) (1, 2). Because ephrin-A is anchored in the plasma membrane by a glycosylphosphatidylinositol linkage and ephrin-B has membrane-spanning domains, the Eph/ephrin system provides complicated bidirectional signaling between adjacent cells that individually express at least one of the family members. Experimentally, tyrosine kinase activity of EphA is activated to transmit forward signals by exogenously applied soluble ephrin-A-Fc fusion proteins (extracellular domain fused to Fc portion of immunoglobulin), whereas reverse signals can be transmitted by glycosylphosphatidylinositol-anchored ephrin-A when activated by exogenous EphA-Fc fusion proteins.

The Eph/ephrin system has been demonstrated to play essential roles in many biological circumstances, including neuronal pathfinding, boundary formation, and angiogenesis in development and tumor progression (1, 2). Although EphA1 is the founding member of the Eph family, its biological functions remain to be elucidated. EphA1 is physiologically expressed in epithelial tissues, and pathological overexpression has been reported in a variety of tumors, including carcinomas of lung, liver, and prostate (2). Artificial overexpression in fibroblasts could be tumorigenic in nude mice. The closest homology is found in EphA2. Breast cancer progression is dependent on both EphA2 activation and ephrin-A1 expression in tumor cells (3). Ephrin-A1 activates EphA2 that is also abundantly expressed in angiogenic endothelial cells, which eventually facilitates metastasis (4). All of this information suggests possible involvement of EphA1 in cancer.

Cellular functions of Eph/ephrin include a proper balance between repulsion from and adhesion to other cells as well as extracellular matrix (ECM)2 such as fibronectin by affecting integrin functions either positively or negatively, depending on different settings (5–7). Fibronectin plays an essential role in cell adhesion and migration and is secreted as a soluble homodimeric protein of 250 kDa (plasma fibronectin) but forms insoluble fibrillar networks in ECM (cellular fibronectin). It is composed of three types of repeating modules, type I, II, and III. An angiogenic inhibitor anastellin is the first type III repeat sequence capable of binding to the amino-terminal type I repeat sequences (8, 9).

Interestingly, genetic engineering studies in Caenorhabditis elegans and mice have shown that Eph has kinase-dependent and -independent functions (10, 11). For example, EphB3 catalytic activity is required for inhibition of integrin-mediated cell adhesion in colorectal epithelial cells. However, inhibition of directional cell migration is totally kinase-independent (12). In addition, both EphB6 and EphA10 appear to lack catalytic activity (13, 14), further supporting the idea that extracellular domain-dependent but kinase-independent functions of Eph might exist.

Although precise regulatory mechanisms of Eph/ephrin expression still remain unknown, regulation by HIF-1α, p53, or lipopolysaccharide in EphA2 (15–17) and that by VEGF or tumor necrosis factor-α in ephrin-A1 (18, 19) have been reported. We have recently shown endothelial tubulogenesis that depends on an ATF/CREB family transcription factor ATF3 (20). It is an immediate early gene induced by a variety of stress signals in neither a tissue- nor stimulus-specific manner. Recent studies have demonstrated that lipopolysaccharide can activate p38, which in turn induces ATF3 (21, 22). VEGF does not activate ATF3, nor does ATF3 stimulate VEGF expression (20). ATF3 forms a heterodimeric complex with another

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2 The abbreviations used are: ECM, extracellular matrix; mAb, monoclonal antibody; GST, glutathione S-transferase; HRP, horseradish peroxidase; DXR, doxorubicin hydrochloride; PBS, phosphate-buffered saline; siRNA, short interfering RNA; CHO, Chinese hamster ovary; VEGF, vascular endothelial growth factor; TRITC, tetramethylrhodamine isothiocyanate; Fn, fibronectin; CREB, cAMP-response element-binding protein; PAI, plasminogen activator inhibitor.
leucine zipper transcription factor AP-1 but not C/EBP family members. This enables activation of promoters that contain binding motifs for AP-1 but not ATF3 itself, as exemplified by the promoter for plasminogen activator inhibitor (PAI-1) we have shown previously (20). In addition, ATF3 is induced by ischemia and hypoxic conditions that are frequently encountered by cancer cells in the tumor microenvironment (23, 24).

In this study, we show an ATF3-dependent expression of EphA1, which exerts a previously unrecognized function in tumor angiogenesis in a kinase-independent manner.

**EXPERIMENTAL PROCEDURES**

**Molecular Reagents**—Mouse monoclonal antibody (mAb) against human EphA1 and all the Fc fusion proteins were purchased from R & D Systems; mouse mAb against glutathione S-transferase (GST), rabbit polyclonal antibody against EphA2 (C-20), ephrin-A1 (V-18), and ATF3 (H-90) from Santa Cruz Biotechnology; rat mAb against CD31 (MEC13.3) was from Pharmingen; rabbit polyclonal antibody against Fn was from LSL; mouse mAb against actin (mAb1501) was from Chemicon; pGEX-4T-1 and pGEX-5X-3, HRP-conjugated sheep anti-mouse Ig, HRP-conjugated goat anti-rabbit Ig, and HRP-conjugated protein A were from GE Healthcare; TRITC- and fluorescein isothiocyanate-conjugated secondary antibodies were from Jackson ImmunoResearch; Matrigel™ Matrix was from BD Biosciences; human plasma fibronectin and cellular fibronectin were from Sigma; doxorubicin hydrochloride (DXR) was from Calbiochem.

**Protein Binding Assays on Microtiter Plates**—Microtiter plates (Nunclon™; NUNC NS, Roskilde, Denmark) were coated with 60 mM of plasma fibronectin, cellular fibronectin, and recombinant fibronectin fragments (I 10–12, I 10–11, and I 12) in PBS and incubated at 4 °C overnight. The following steps were then performed at room temperature. The plates were washed with PBS and treated for 2 h with blocking buffer (4% bovine serum albumin in PBS). The plates were subsequently washed three times with PBS and incubated with various concentrations of Fc chimeras of EphA, EphB4, ephrin-A1, and ephrin-B2 diluted with PBS. The plates were again washed three times and then incubated for 1 h with HRP-conjugated protein A (1:1,000) in blocking buffer. After washing in PBS, the plates were allowed to react with 3,3',5,5'-tetramethylbenzidine substrate reagent (BD Biosciences); color development was stopped by the addition of 1 n H2SO4. The absorbance at 450 nm was determined using a microplate reader. Competitive binding assay of Fn fragments and EphA1 to plasma Fn was performed as follows: microtiter plates coated with plasma fibronectin at 60 nM were washed with PBS, blocked with blocking buffer, and incubated EphA1-Fc (150 nM) with various concentrations of fibronectin fragments (234–7500 nM of I 10–12 and I 12) diluted with PBS.

**In Vitro Angiogenesis Assay in Matrigel**—In vitro angiogenesis assay was performed as described previously (20). Briefly, cells were plated at 3 × 10^4 per well onto Matrigel and then incubated with 1 μg/ml Fc chimeric proteins and 40 μM recombinant fibronectin fragments. Branching images were captured using an Olympus IX70 microscope and digital camera. Digital images were processed using Adobe Photoshop 5.0 LE software. Quantification of the total length of cords in branching morphogenesis was performed using Scion Image software. Means of the results from six independent experiments were calculated.

**siRNA-mediated Knockdown Experiments**—Based on rat EphA1 sequence, sense primer (5’-CCUGUGAGAAGUGGCAUUATT-3’) and antisense primer (5’-UAAUGUCCAUU-CUCACAGGTA-3’) were designed. Annealing reactions were performed with sense and antisense to give EphA1-specific siRNA duplexes, and with sense/sense as a control. We also used a nontargeting control siRNA against Nox1 that was previously shown to have no effect on the ATF3-dependent *in vitro* angiogenesis (20). Cells were seeded at 2 × 10^4 per well 24 h before transfection using TransIT-TKO (Mirus). After incubation for 24 h, cells were plated at 3 × 10^4 per well onto Matrigel.

**RNA Preparation and Southern Blotting**—Total RNA was prepared from rat lung using ISOGEN (NIPPON GENE), following the manufacturer’s instructions. cDNAs were reverse-transcribed from 10 μg of rat lung total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). PCR primers, 5’-CATGTACACGACTGCCCA-3’ and 5’-CTGATCTGTCATGAGGAG-3’, were used to amplify the 254–775 region of rat EphA1 (GenBank™ accession number XM_231658), and PCR primers 5’-GGTACACGAGATCCACC-3’ and 5’-AGCATTCATCATGATGTTG-3’ were used to amplify the 1505–1941 region of rat EphA2 (GenBank™ accession number XM_001072610). The specificity of the PCR-amplified products was further verified by Southern hybridization. Probe labeling, hybridization, and detection were performed by using ECL direct acid labeling and detection systems (GE healthcare) following the manufacturer’s instructions.

**Reporter Assay**—PCR primers, 5’-GACTGTCGATGATGCTACACTG-3’ and 5’-CCAGGGGCGGCAGCGCTCTCC-3’, were used to amplify the −1027 to +82 region of the rat EphA1 promoter (GenBank™ accession number NM_047690). The PCR product was subcloned into SmaI/HindIII sites of the pGL3 basic vector (Promega) to generate pEphA1-Luc. The luciferase construct of the ephrin-A1 promoter was generated in a similar fashion with primers 5’-TGGGCAAGGTTCTGTGAG-3’ and 5’-AAGCTCGAGGTAGGTTCGTC-3’, the PAI-1 promoter-luciferase fusion reporter construct pGL-3-PAI-1/full (−829 to +36) was described previously (20). NP3/ATF3-Tet cells were seeded at 2 × 10^5 per well 24 h before ATF3 induction. In every case, 50 ng of pRL-SV40 (Promega) was added as an internal control. Cells were harvested 24 h after transfection using DMRIE-C (Invitrogen), and luciferase activities were determined with the dual luciferase reporter assay system (Promega). Means of the results from six independent experiments were calculated.

**Yeast Two-hybrid Assay**—Yeast two-hybrid assay was performed as described previously (25).

**CHO Cell Culture and Transplantation**—CHO cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For EphA1-Fc overexpresser constructs, the extracellular region (amino acids 1–1635, GenBank™ accession number NM_005232) and human IgG2 region (amino acids 1–696, GenBank™ accession number NM_005232)
Fibronectin Fragment Binds EphA1

NM_001040070 were generated by PCR and cloned in-frame into the HindIII and XhoI site of pcDNA3 (Invitrogen). CHO cells were transfected using Effectene (Qiagen) and were cultured in 400 μg/ml of genetin (Sigma). For transplantation experiments, 10⁶ cells were suspended in 100 μl of PBS. The suspension was injected subcutaneously into mice using a 1-ml syringe and a 25-gauge needle. Tumors derived from these injected cells were surgically collected from the mice 14 days after implantation.

Matrigel Plug Assay—Five- to 6-week-old C57BL/6 mice were used. Matrigel was mixed with 100 ng/ml VEGF (Sigma). The Matrigel mixtures (500 μl) containing 16 μg/ml mock, EphA1-Fc, or I12 were injected subcutaneously, and mice were sacrificed 7 days after transplantation, and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were either directly frozen for cryostat sections for anti-CD31 staining.

Statistical Analysis and Analysis of the Binding Data—Data are expressed as means ± S.D. Comparisons between two groups were performed with the two-tailed, paired Student’s t test and the Mann-Whitney U test. In all analyses, p < 0.05 was considered statistically significant. The binding affinity constants were estimated following nonlinear regression analysis of the saturation curves using the software GraphPad Prism, version 4 (GraphPad Software).

Purification of Recombinant Fibronectin Fragment Proteins—The fibronectin cDNAs 6280–7053 (I 10–12), 6280–6750 (I 10–11), and 6751–7053 (I 12) were cloned into pGEX-4T-1. The proteins were expressed in Escherichia coli and purified with a glutathione column. The purified GST fusion proteins were treated with thrombin to remove GST, dialyzed against PBS, and stored.

RESULTS

EphA1 Is Up-regulated in an ATF3-dependent Manner—We have established a biological system in which tetracycline-regulated expression of ATF3 in NP31 endothelial cells (NP31/ATF3-Tet) caused angiogenesis in vitro (20) (Fig. 1A). Looking for ATF3-dependent angiogenesis-related proteins, we happened to find a 5-fold up-regulation of EphA1 (Fig. 1A) (26). There was almost no change in expression levels of ephrin-A1 and EphA2 on which VEGF depends for angiogenesis (Fig. 1A) (18, 27). Reverse transcriptase (RT)-mediated PCR and subsequent Southern blot analysis revealed that EphA1 is up-regulated also at mRNA levels in an ATF3-dependent manner, whereas no change was observed in EphA2 (Fig. 1B).

To further ensure the promoter activity, we generated a set of promoter constructs, including ΔCREB with a subtle deletion of the −1546 CREB site and large deletion mutants (supplemental Fig. 2). We also isolated 743-bp ephrin-A1 promoter region that contains a single AP-1 site at 288. Tetracycline withdrawal alone repeatedly induced ~1.2- to ~1.5-fold increase of luciferase activity in vector alone for unknown reasons. However, as shown in supplemental Fig. 2, a deletion of the −946 CREB site significantly reduced the promoter activity of EphA1. No significant increase in the ephrin-A1 promoter activity was found in this assay.

EphA1 Is Required for in Vitro Angiogenesis—To investigate whether the Eph/ephrin system affects endothelial tubulogenesis that depends on ATF3, we performed in vitro angiogenesis assay with NP31/ATF3-Tet cells in the presence of recombinant Fc fusion proteins of the extracellular domain of Eph or anti-EphA1 siRNAs. Anti-EphA1 siRNA but not control RNAs
Fibronectin Fragment Binds EphA1

**FIGURE 2. EphA1 is required for in vitro angiogenesis.** A, total cell lysates from cells treated with anti-EphA1 siRNAs, including (sense/sense) SS at 40 nM (lane 1), (antisense/antisense) AS at the indicated doses (lanes 2–4), or an irrelevant siRNA negative control (AS*, anti-Nox1 siRNAs, lane 5) (20), were subjected to αEphA1 (upper panel) and α-actin (lower panel) Western blotting. Note a dose-dependent knockdown. B, in vitro angiogenesis assay in the presence of no treatment (mock), 1 μg/ml of the indicated Fc fusion proteins, or anti-EphA1 siRNAs at the indicated concentrations. Relative values to the mock were shown as mean ± S.D. from six independent experiments.

efficiently knocked down EphA1 protein levels in a dose-dependent manner (Fig. 2A). The inhibition of ATF3-induced transactivation of EphA1 by the siRNA abrogated angiogenesis by roughly 70% at 80 nM as compared with control RNAs (Fig. 2B and supplemental Fig. 3). Exogenously applied EphA1-Fc can bind ephrin-A1 that is endogenously expressed in cells to activate reverse signaling. However, EphA1 occupied by EphA1-Fc can no longer bind endogenous EphA1 or EphA2 to exert forward signaling. Both EphA1-Fc and EphA2-Fc failed to inhibit angiogenesis, suggesting that none of their forward signaling is required (Fig. 2B). Although EphB4 and ephrin-B2 have also been shown to regulate angiogenesis (28), Fc fusion proteins for either of these had almost no effect in our angiogenesis assay. These results suggest that the expression of EphA1 but not its forward signaling is required, underscoring the functional significance of the extracellular domain of EphA1. One such function is the EphA-to-ephrin-A1 signaling. Ephrin-A1-Fc can block the reverse signaling by occupying the endogenous EphA1 and EphA2. As shown in Fig. 2B, ephrin-A1-Fc inhibited the angiogenesis by 55%, which is seemingly consistent with the idea that the EphA1-to-ephrin-A1 reverse signaling may play a positive role in the angiogenesis. However, ephrin-A1-mediated reverse signaling could take place by the constantly expressed endogenous EphA2 even before ATF3-induced tubule formation. Therefore, EphA1-specific reverse ephrin-A1 signaling and/or a still unknown function of the extracellular domain of EphA1 might exist.

Doxorubicin-induced ATF3 Activation Causes Endothelial Tubulogenesis—To show the involvement of ATF3 in angiogenesis in more physiological conditions, we treated the nontubulogenic endothelial cell line NP31 (29) with doxorubicin. Doxorubicin has been reported to induce ATF3 expression in MCF7 breast cancer cells (30). NP31 cells tolerated doxorubicin at a 62 nM to 1 μM concentration in the usual tissue culture conditions, and ATF3 activation was similarly observed at those doses, which was concomitant with increased expression of EphA1 (Fig. 3A). Although NP31 cells were unable to survive in Matrigel against 500 nM doxorubicin, we could repeatedly observe the cells without apoptosis at 125 nM for 16–48 h. Although the level of cord formation was ~25~30% of that in ATF3-induced tubulogenesis as reported previously (20), ATF3-mediated expression of EphA1 by doxorubicin induced tubulogenesis appreciably (Fig. 3B).

Fibronectin Type I Repeat Protein Binds EphA1—This prompted us to search for proteins capable of interacting with the extracellular domain. A bait designed to encode the extracellular domain was used to screen a human placenta yeast two-hybrid library (25). We found four independent clones that encode overlapping carboxyl-terminal fibronectin fragments. The shortest contained amino acids 2094–2351 including type I repeat numbers 10–12 (I 10–12) (Fig. 4A). Because the yeast two-hybrid system also gave a positive...
**Fibronectin Fragment Binds EphA1**

**A**

| Type I | Type II | Type III | Type I |
|--------|---------|----------|--------|
| 12345 6 7 8 9 | 2345 6 7 8 9 | 10112 | 2345 6 7 8 9 |

Prey

**B**

Prey:

Bait:

SD-2

SD-4

**FIGURE 4.** EphA1 binds fibronectin fragments. A, schematic structure of the full-length fibronectin. Note that I 3–5 is amino acids 225–360; III 14–15 is amino acids 1906–2073; spacer is amino acids 2082–2238; and I 10–12 is amino acids 2241–2421 (GenBank™ accession number NM_212475). B, yeast two-hybrid assay to test binding of prey (–, vector alone) and bait (E, extracellular domain of EphA1; B, BCR fragment (25), an irrelevant bait control) in synthetic medium SD (SD-2, expression of prey/bait genes; SD-4, their positive interaction).

**FIGURE 5.** Fibronectin type I repeat proteins bind EphA1. A, B, and D, increasing concentrations of EphA1-Fc, ephrin-A1-Fc, or Fc alone (150–1200 nM) were incubated with full-length plasma fibronectin (pFn) (A), cellular fibronectin (cFn) (B), or I 10–12-coated (60 nM) microtiter wells. C, Coomassie staining of the E. coli-expressed and purified recombinant type I repeat proteins, 2 µg each. E and F, binding of I 10–11 (E) or I 12 (F) to the indicated series of EphA1 (1 to 6)-Fc, EphB4-Fc, ephrin-B2-Fc, and ephrin-A1-Fc. Increasing concentrations of Fc fusion proteins or Fc alone (234–150 nM) were incubated with I 10–11-coated (60 nM) microtiter wells. Bound Fc fusion proteins were detected by incubation with HRP-conjugated protein A. Binding ability was found only EphA1-Fc, EphA2-Fc, and EphA8-Fc but not in EphA3 to 7-Fc, EphB4-Fc, ephrin-A1-Fc, ephrin-B2-Fc, and Fc alone.

**FIGURE 5.**

signal for binding of the bait to the amino-terminal I 3–5 but not III 14–15 or the spacing sequence between III-15 and I-10 (Fig. 4, A and B), we have decided to focus on I 10–12 in the subsequent experiments.

To test if the full-length fibronectin directly interacts with the extracellular region of EphA1, we conducted solid-phase binding assays with either purified plasma or cellular fibronectin (Fig. 5, A and B) immobilized to microtiter plates. A direct examination by enzyme-linked immunosorbent binding assay revealed that the full-length fibronectin (Fn) was capable of binding to EphA1-Fc and surprisingly to ephrin-A1-Fc as well (Fig. 5A). We could repeatedly observe the binding of fibronectin to Fc alone. The binding affinity is low but appreciable with \( K_d \) 137 and 263 nM with plasma and cellular fibronectin, respectively, for EphA1-Fc.

We then expressed I 10–12, I 10–11, and I 12 in the form of GST fusion proteins in E. coli and purified them by removing GST by protease-mediated cleavage (Fig. 5C). Purified I 10–12 showed a decreased affinity toward EphA1 with \( K_d \) 417 nM, but it totally lost its ability to bind ephrin-A1 or Fc alone (Fig. 5D). To our surprise, both I 12 and I 10–11 showed a drastic enhancement of its affinity toward EphA1-Fc (\( K_d \) 96 and 80 nM, respectively) (Fig. 5, E and F). When a series of EphA (2–8)-Fc, EphB4-Fc, and ephrin-B2-Fc were included in the assays, the highest affinities were found with EphA2-Fc (\( K_d \) 18 and 19 nM, respectively). Although EphA8-Fc also showed appreciable affinities, the rest of the Fc fusion proteins showed negligible affinities in both cases.

**Inhibition of in Vitro Angiogenesis by Fibronectin Type I Fragments—**

Matrigel that we used in the *in vitro* angiogenesis assay contains ECM proteins type IV collagen and laminin, but our endothelial cells produce fibronectin by themselves (Fig. 6A). The a5β1 integrin is the fibronectin receptor, and anti-β1 blocking antibody is capable of abrogating the angiogenesis (29). Therefore, we tested if the *in vitro* angiogenesis is also dependent on the binding between the extracellular domain of EphA1 and fibronectin by utilizing the purified fibronectin fragments shown in Fig. 5. I 10–12 inhibited ATF3-dependent angiogenesis in a synergistic fashion with ephrin-A1-Fc but not with EphA1-Fc (Fig. 6B). Curiously, however, neither I 10–11 nor I 12 retained the inhibitory activity despite their high affinity to EphA1-Fc. Solid-phase competition assays

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**TABLE**

| Protein | I 10-12 | EphA1-Fc | EphA2-Fc | EphA8-Fc | ephrin-A1-Fc |
|---------|---------|-----------|-----------|-----------|--------------|
| Proteins (nM) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A 450 nm | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

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revealed that I12 could no longer block the binding between Fn and EphA1-Fc but I10–12 could in a dose-dependent manner (Fig. 6C).

Both EphA1-Fc and I12 Inhibit VEGF-dependent Angiogenesis in Vivo—To test if EphA1-Fc and fibronectin bind in vivo, we established CHO cells secreting EphA1-Fc (Fig. 7A) and subcutaneously transplanted them into nude mice. Immunostaining revealed merged images of EphA1 with fibronectin (Fig. 7B). Fibronectin can be cleaved into fragments of various sizes by proteases. Protein A-Sepharose-bound EphA1-Fc proteins from the nude mice tumors (Fig. 7C), but not from CHO/EphA1-Fc cells cultured in vitro (Fig. 7D), contained a naturally occurring fibronectin fragment around 40 kDa (Fig. 7C, arrow). Interestingly, expression of EphA1-Fc suppressed tumor growth, which was accompanied by decreased angiogenic activity as judged by CD31-positive staining (Fig. 8).

Because I12 acquired a high affinity to EphA2, we were curious to examine its effects on the VEGF-dependent angiogenesis in Matrigel plug assay. Both I12 and EphA1-Fc could inhibit vascular invasion into Matrigel as judged by quantified vascular area as well as CD31-positive signals (Fig. 9).

**DISCUSSION**

VEGF-dependent in vitro angiogenesis is reported to be blocked by recombinant proteins containing the extracellular domain of EphA2 fused to the Fc portion of immunoglobulin (EphA2-Fc) (18), ephrin-A1-Fc (31), and EphB4-Fc (28). Our

**FIGURE 7. Secreted EphA1-Fc binds fibronectin in vivo.** A, anti-EphA1 Western blotting of protein A-Sepharose (PAS)-bound fraction of conditioned medium and whole cell lysates (WCL) of CHO cells secreting EphA1-Fc or mock. Immunoblotting (IB) was carried out using anti-EphA1 antibody. B, anti-EphA1 and anti-fibronectin (Fn) immunostaining of nude mice tumors derived from CHO/mock and CHO/EphA1-Fc cells. Bar, 100 μm. C and D, anti-fibronectin Western blotting of whole cell lysates (WCL) and protein A-Sepharose (PAS)-bound fraction of the whole cell lysates from CHO/mock and CHO/EphA1-Fc cells in vitro culture (vitro, D) or from nude mice tumors derived from those cell lines (vivo, C), plasma Fn (pFn, 20 ng/ml), and of purified GST-I10–12, GST-I11–12, and GST-I10–12 proteins (20 ng/ml).

result that expression of EphA1 but not its signaling is required in the ATF3-dependent angiogenesis led us to uncover fibronectin-binding function of the extracellular domain of EphA1. Competitive inhibition of the binding by the fibronectin fragment I10–12 resulted in the abrogation of the angiogenesis.

I10–12 and I1–5 correspond to the extensively studied carboxyl-terminal 19-kDa and amino-terminal 29-kDa fibrin-binding fragments, respectively. The dissociation constant between E. coli-expressed monomeric form of I10–12 and EphA1-Fc showed a low affinity of 417 nM. The $K_d$ values between fibrin $\alpha$C-fragment and monomeric 19 kDa or its dimeric forms were 3.1 μM and 360 nM, respectively (32). Another well known protein that binds the 29-kDa fragment is
Fibronectin Fragment Binds EphA1

The highest binding ability of I12 was found with EphA2 (KD ≈ 18 nM), which is comparable with the level of KD values for ephrin-A1 binding to EphA1 (KD ≈ 267 nM) and EphA2 (34). I10–11 behaved in a similar manner (Fig. 5E). This makes a good contrast with fibrin binding where the full-length fibronectin has a higher affinity of KD 2.1 nM than the type I repeat fragments (35). Considering that I10–11 can no longer bind to fibrin, the property of Eph binding appears to depend on the individual type I repeat.

One of the interesting features of Eph/Fn binding is that none of the fibronectin fragments (I10–12, I12, and I10–11) could induce autophosphorylation, endocytosis of EphA1, or cell growth in 293 cells stably overexpressing EphA1. Nor could they alter these biological activities in the presence of ephrin-A1.3 An angiogenic inhibitor anastellin is the first type III repeat sequence capable of binding to the amino-terminal type I repeat sequences (8, 9). In addition, Eph receptors contain fibronectin type III repeat sequences carboxyl-terminal to the ephrin-binding domain (1). However, the gross difference in binding of I12 to a series of Eph family members suggests targeting specificity among Eph proteins. In our ATF3-dependent in vitro angiogenesis, EphA2 could be necessary but is certainly not sufficient because it is already expressed even before the cells acquire tubulogenic ability (Fig. 1A). The ephrin-A1-to-EphA2 forward signaling is not required (Fig. 2B). Even if I12 with a much higher binding affinity to EphA2 than EphA1 (Fig. 5F) could block certain EphA2 function(s), it failed to affect the angiogenesis (Fig. 6B). However, I12 attenuated vascular invasion to VEGF-containing Matrigel (Fig. 9). We assume that the tight engagement of I12 with EphA2 could modify its regulatory function(s) in VEGF-dependent angiogenesis.

Tumor angiogenesis has been reported to be governed primarily by VEGF and inhibited in EphA2 knock-out mice or by application of EphA2-Fc but not EphA1-Fc (27, 36, 37). Given no expression of EphA1 in CHO cell-derived nude mice tumors (Fig. 7), we assume that the essential Eph in endothelial cells in VEGF-dependent tumor angiogenesis is EphA2 or EphB4 but not EphA1. However, EphA1-Fc could inhibit both tumor angiogenesis (Fig. 8) and Matrigel invasion (Fig. 9). It is possible that binding of EphA1-Fc to the endogenous ephrin-A1 could indirectly block ephrin-A1-to-EphA2 signaling. Therefore, we suppose that I12 might be useful for blocking tumor progression.

Antiangiogenic cryptic fragments that are usually buried in the full-length endogenous proteins are exposed to exert its activity after cleavage, including endostatin, platelet factor-4, tumstatin, thrombospondin, prolactin, and so forth (38). Detection of an EphA-binding fibronectin fragment in vivo suggests that antiangiogenic switching by certain proteases may

3 J. Masuda, T. Yamazaki, and Y. Maru, unpublished observations.
exist in physiological and pathological circumstances. Two fibronectin-derived angiogenic inhibitors are known, anastellin (III-1) and RGD from III-9. The mechanism of inhibition is disturbance of the interaction between ECM and its integrin receptor. Both RGD and anti-β1 integrin antibody were also capable of blocking the ATF3-dependent tubulogenesis (29 and data not shown).

Elucidation of biological significance of the interaction between the extracellular domain of EphA1 and fibronectin or its fragments still needs further investigation. One possibility might be modification of integrin-mediated cell adhesion or cell adhesion by itself independently of integrin. RGD belongs to the former category. In addition to outside-in integrin signaling, fibronectin might stimulate EphA1 without tyrosine kinase activation within the cells or modify its extracellular domain that is responsible for ephrin-A1-mediated reverse signaling.

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