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# Stable interaction between $\alpha 5\beta 1$ integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1

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**D**uring angiogenic remodeling, Ang-1, the ligand of Tie2 tyrosine kinase, is involved in vessel sprouting and stabilization through unclear effects on nascent capillaries and mural cells. In our study, we hypothesized that the Ang-1/Tie2 system could cross-talk with integrins, and be influenced by the dynamic interactions between extracellular matrix and endothelial cells (ECs). Here, we show that  $\alpha 5\beta 1$  specifically sensitizes and modulates Tie2 receptor activation and signaling, allowing EC survival at low concentrations of Ang-1 and inducing persistent EC motility. Tie2 and  $\alpha 5\beta 1$  interact

constitutively;  $\alpha 5\beta 1$  binding to fibronectin increases this association, whereas Ang-1 stimulation recruits p85 and FAK to this complex. Furthermore, we demonstrate that Ang-1 is able to mediate selectively  $\alpha 5\beta 1$  outside-in FAK phosphorylation. Thus, Ang-1 triggers signaling pathways through Tie2 and  $\alpha 5\beta 1$  receptors that could cross-talk when Tie2/ $\alpha 5\beta 1$  interaction occurs in ECs plated on fibronectin. By using blocking antibodies, we consistently found that  $\alpha 5\beta 1$ , but not  $\alpha v\beta 3$  activation, is essential to Ang-1-dependent angiogenesis *in vivo*.

## Introduction

During vertebrate development, angiogenesis identifies a phase of remodeling and maturation of the primary capillary plexus (Yancopoulos et al., 2000). In the adult life, angiogenesis occurs in physiologic and pathologic conditions in which transport of oxygen and nutrients are needed (Folkman, 1995). The maturation of the vascular network is regulated by extracellular matrix (ECM) remodeling and by proliferation, survival, apoptosis, and motility of endothelial cells (ECs). A balanced activation of growth factor and adhesive receptors is instrumental for physiologic remodeling; perturbation of this homeostasis results in the establishment of a chaotic vasculature (Stupack and Chersesh, 2002).

Ang-1 is the ligand of the endothelial tyrosine kinase receptor, Tie2 (Davis et al., 1996). Mice lacking Ang-1 die during embryo development (E12.5) showing a poorly remodeled

and mature vasculature with defects in EC adhesion and spreading to the underlying ECM (Suri et al., 1996). The role of Ang-1 in adult angiogenesis is controversial. Several investigators have shown that Ang-1 acts as proangiogenic factor, whereas others have demonstrated the opposite (Suri et al., 1998; Chae et al., 2000; Hangai et al., 2001; Hawighorst et al., 2002; Shim et al., 2002; Uemura et al., 2002; Stoeltzing et al., 2003). However, *in vitro* Ang-1 promotes a proangiogenic program in ECs characterized by expression of metalloproteases and plasmin, and induction of morphogenesis, motility, and survival (Koblizek et al., 1998; Papapetropoulos et al., 1999; Cascone et al., 2003a; Das et al., 2003). It recently was demonstrated that Ang-1 promotes cell adhesion (Arai et al., 2004; Lemieux et al., 2005), and that this process is mediated by  $\alpha 5$ -integrin in ECs (Carlson et al., 2001). Moreover, the finding that Ang-1 can bind ECM extracts from carcinoma cells (Xu and Yu, 2001) has offered new insights to understand the role of Ang-1 in modulating the angiogenic microenvironment.

Cell adhesion is mediated by integrin heterodimers (Giancotti and Ruoslahti, 1999). Cross-talks between integrins and growth factor receptors were shown to coordinate biologic processes through the regulation of downstream and inside-out signaling pathways (Schneller et al., 1997; Soldi et al., 1999; Byzova et al., 2000; Sieg et al., 2000; Baron et al., 2002; Lee and Juliano, 2002). Tyrosine kinase receptors and integrins share many down-

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Abbreviations used in this paper: Ab, antibody; anti-CBP, anti-fibronectin cell binding peptide mAb; CAM, chick chorioallantoic membrane; EC, endothelial cell; ECM, extracellular matrix; MTT, [(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]; PAE, porcine aortic endothelial; PI3-K, phosphatidylinositol-3-kinase; VEGF, vascular endothelial growth factor. VEGFR, vascular endothelial growth factor receptor.

The online version of this article contains supplemental material.

Supplemental Material can be found at:  
<http://jcb.rupress.org/content/suppl/2005/09/13/jcb.200507082.DC1.html>

stream effectors. In particular, activated Tie2 recruits p85, phosphorylates FAK, and modulates Rho GTPases (Kontos et al., 1998; Jones et al., 2001; Cascone et al., 2003a), which also participate in outside-in integrin signaling (Hood and Cheresh, 2002).

Integrins have crucial roles in angiogenesis (Hodivala-Dilke et al., 2003) and allow vascular cells to adapt their adhesive machinery to the so-called “provisional” ECM components, like fibronectin, collagen, and vitronectin, that are exposed by basement degradation around sprouting vessels (Kalluri, 2003). Integrins  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha 2\beta 1$ , and  $\alpha 5\beta 1$  are up-regulated in newly formed blood vessels (Max et al., 1997; Kim et al., 2000b), and  $\alpha v\beta 3$  and  $\alpha v\beta 5$  antagonists inhibit *in vitro* and *in vivo* angiogenesis (Brooks et al., 1995; Drake et al., 1995; Hammes et al., 1996).  $\alpha 2$ -blocking antibodies (Abs) inhibit vascular endothelial growth factor (VEGF)-A-induced angiogenesis (Senger et al., 1997). Vascular defects are described in  $\alpha 5$ -null embryoid bodies and teratocarcinomas (Taverna and Hynes, 2001; Francis et al., 2002); antagonists of the central cell-binding domain of fibronectin also inhibit angiogenesis (Kim et al., 2000b). Integrins can exist in different functional states that regulate their biologic functions (Hynes, 2002). *In vivo* integrin activity depends on the extracellular environment; it has been shown that modulation of ECM concentration and patterning leads to different cell responses ranging from apoptosis to growth and differentiation (Dike et al., 1999).

Here, we hypothesize that Ang-1/Tie2 could mediate different biologic effects under the influence of integrin activity. We demonstrate that Tie2 and  $\alpha 5\beta 1$  form a constitutive and specific complex, and that Ang-1/Tie2 system is sensitized by  $\alpha 5\beta 1$  engagement to fibronectin. Furthermore, we show that  $\alpha 5\beta 1$  function is essential to mediate *in vivo* Ang-1-dependent angiogenesis in a chick chorioallantoic membrane (CAM) assay.

## Results

### Fibronectin sensitizes Tie2 activation and signaling to low Ang-1 concentrations

Differently from other tyrosine kinase receptor ligands, Ang-1 does not induce cell proliferation. Instead, it delays EC death triggered by different cellular stresses by inducing phosphatidylinositol 3-kinase (PI3-K)/AKT signaling activation (Kim et al., 2000a).

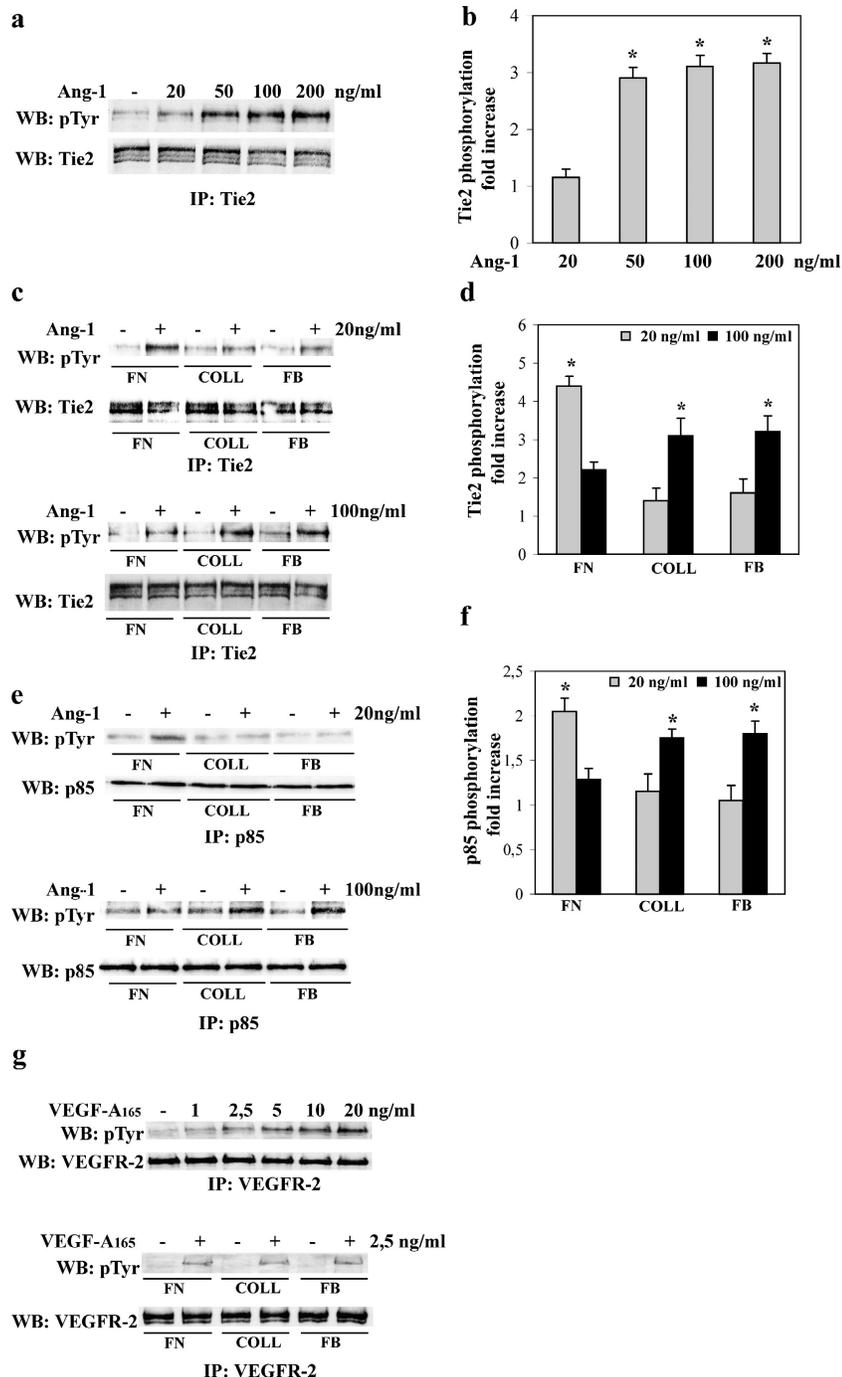
We analyzed the influence of the integrin function on Ang-1-mediated EC survival; plating ECs on ECM largely involved in angiogenesis, such as fibronectin, collagen I, and fibrinogen, ligands for  $\alpha 5\beta 1$ ,  $\alpha 2\beta 1$  and  $\alpha 1\beta 1$ , and  $\alpha v\beta 3$  integrins, respectively. ECs were starved and kept for 24 h in 0.5% FCS in the presence of increasing concentrations of Ang-1 (Fig. S1 a; available at <http://www.jcb.org/cgi/content/full/jcb.200507082/DC1>). In cells plated on collagen I or fibrinogen, the survival effect of Ang-1 reached relevant values at 200 ng/ml, and the plateau condition at 500 ng/ml. When fibronectin was used as substrate, Ang-1 was effective at 100 ng/ml and attained the plateau at 200 ng/ml. These results demonstrated a shift of the dose-response of EC survival on fibronectin versus lower Ang-1 concentration. This effect of fibronectin was not observed when ECs were stimulated with VEGF-A<sub>165</sub> (Fig. S1 b).

Because the dose-response effect of Ang-1 on EC survival was dependent on ECM components (Fig. S1 a), we analyzed the effect of specific integrin engagement on Tie2 activation. To verify if ECM could modify Tie2 phosphorylation dose-response, we first established Tie2 phosphorylation in ECs plated on native ECMs (Fig. 1, a and b). Next, on the basis of the curve in Fig. 1 b, we stimulated ECs plated on fibronectin, collagen I, or fibrinogen with an ineffective concentration of Ang-1 (20 ng/ml), and with a higher dose (100 ng/ml) that triggered a 3.15-fold increase of Tie2 phosphorylation (Fig. 1 c). ECs plated on collagen I or fibrinogen showed a Tie2 phosphorylation response (Fig. 1, c and d) similar to ECM native conditions (Fig. 1 b). On fibronectin, ECs underwent a marked Tie2 phosphorylation with 20 ng/ml of Ang-1 (4.4-fold increase), whereas 100 ng/ml was less effective (2.2-fold increase; Fig. 1, c and d). As control, Fig. 1 g shows that the effect of a concentration of VEGF-A<sub>165</sub> that induces a negligible phosphorylation of VEGF receptor (VEGFR)-2 was not affected by ECM proteins, including fibronectin.

Therefore, EC adhesion to fibronectin specifically allowed Tie2 to be responsive to lower amounts of its ligand. We then examined p85 phosphorylation that is recruited by activated Tie2 and mediates PI3-K signaling (Kontos et al., 1998) during Ang-1-dependent cell survival and motility (Kim et al., 2000a; Cascone et al., 2003a). Similarly to Tie2, p85 was phosphorylated at 20 ng/ml Ang-1 on fibronectin (2.0-fold increase) and 100 ng/ml on collagen I (1.75-fold increase) or fibrinogen (1.6-fold increase; Fig. 1, e and f). Therefore, Tie2 activation and signaling were increased at lower Ang-1 concentrations when ECs adhered to fibronectin; this was consistent with the results obtained in cell survival (Fig. S1 a).

### Tie2 interacts selectively and constitutively with $\alpha 5\beta 1$

We investigated whether Tie2 receptor could interact physically with integrins, in particular with  $\alpha 5\beta 1$ , the main fibronectin receptor. Lysates from ECs cultured on native ECM were immunoprecipitated with anti- $\alpha 5\beta 1$  (AB1950) or anti- $\beta 1A$  (Fig. 2 a), anti- $\alpha 2\beta 1$  (BHA2.1) or anti- $\alpha 2\beta 1$  (JBS2) (Fig. 2 b), anti- $\alpha v\beta 3$  (LM609), or anti- $\alpha v\beta 3$  (25E11) (Fig. 2 c) Abs and blotted with anti-Tie2 Ab. Tie2 coimmunoprecipitated with  $\alpha 5\beta 1$ , but not with  $\alpha 2\beta 1$  or  $\alpha v\beta 3$  integrins. VEGFR-2 was not detected in  $\alpha 5\beta 1$  immunoprecipitates (Fig. 2 a). To confirm further the specificity of the association between Tie2 and  $\alpha 5\beta 1$ ,  $\alpha 5\beta 1$  immunoprecipitates were re-immunoprecipitated with anti-Tie2 and blotted with anti-Tie2 Ab (Fig. 2 d). However, Tie2/ $\alpha 5\beta 1$  association was not detectable by blotting Tie2 immunoprecipitation with anti- $\alpha 5\beta 1$  (unpublished data), as it has been described for other integrin/tyrosine kinase receptor complexes (Baron et al., 2002; Woodard et al., 1998). To gain further insights about the specificity of Tie2/ $\alpha 5\beta 1$  interaction, CHO lacking (CHO B2) or expressing (CHO B2a27)  $\alpha 5$  subunit were transfected transiently with Tie2, and immunoprecipitated with an anti- $\beta 1$  Ab (Fig. 2 e). Tie2 coimmunoprecipitated with  $\beta 1$  only in CHO B2a27 cells (Fig. 2 e). Thus, Tie2 interacts selectively and constitutively with  $\alpha 5\beta 1$  and not with other  $\beta 1$  heterodimers. Then, we labeled, by biotinylation, cell



**Figure 1.  $\alpha 5\beta 1$  engagement influences Tie2 phosphorylation and signaling.** ECs cultured on native ECM (a) and (g), or on 5  $\mu\text{g}/\text{ml}$  fibronectin (FN), 5  $\mu\text{g}/\text{ml}$  collagen I (COLL), or 20  $\mu\text{g}/\text{ml}$  fibrinogen (FB) (c, e, and g) were stimulated with Ang-1 for 5 min (a, c, and e) or VEGF-A<sub>165</sub> (g) at the indicated concentrations, lysed, immunoprecipitated (IP) with either anti-Tie2 (a and c), anti-VEGFR-2 (g), or anti-p85 (c) and blotted as indicated. Panels b, d, and f show the densitometric analysis of a, c, and e, respectively, and indicate the fold increase of Tie2 or p85 tyrosine phosphorylation normalized to the total protein amount. Values shown are means  $\pm$  SD of five independent experiments. In b, statistical significance (\*,  $P < 0.01$ ) is shown for increasing Ang-1 concentrations compared with 20 ng/ml Ang-1. In d and f, statistical significance (\*,  $P < 0.01$ ) is shown for Ang-1-stimulated ECs for the indicated substrate.

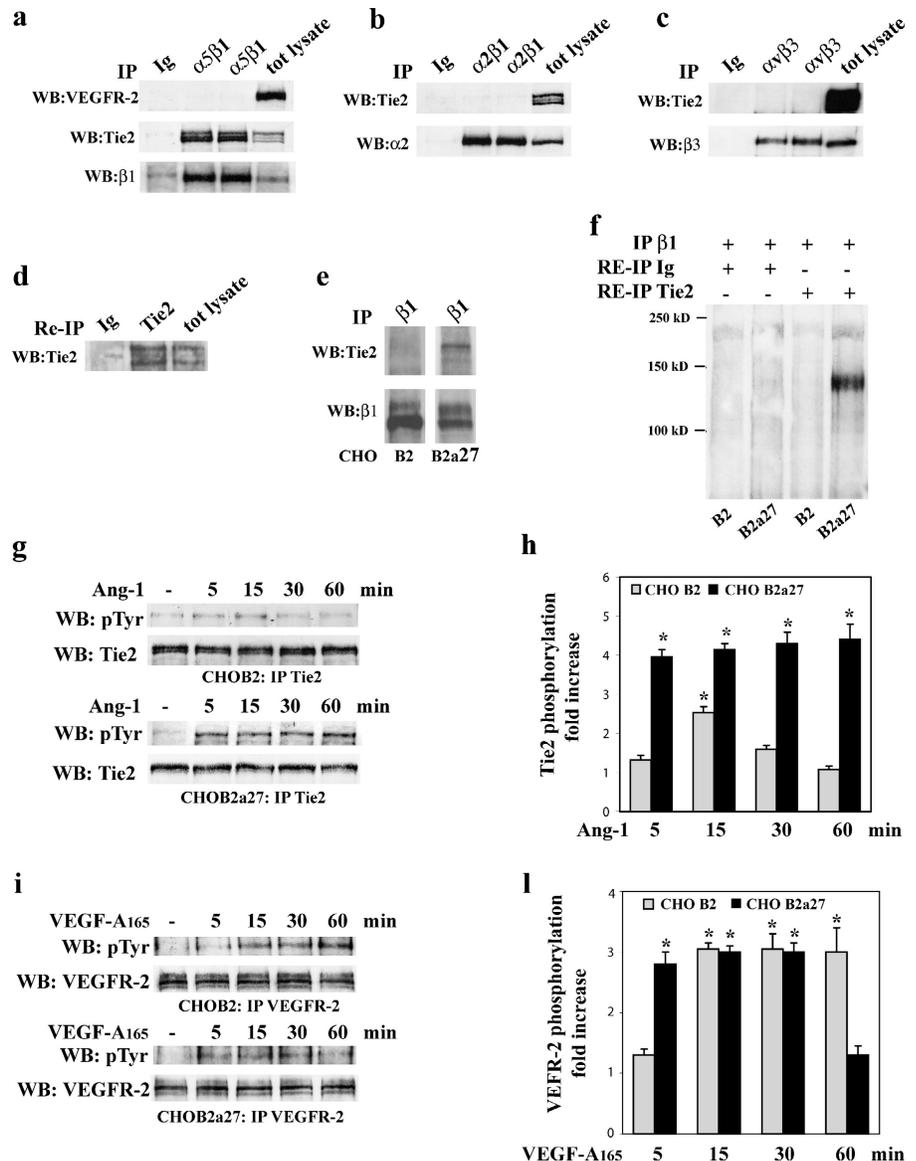
surfaces of CHO B2 and CHO B2a27 expressing Tie2, and we re-immunoprecipitated Tie2 from  $\beta 1$  immunoprecipitates as above. Biotinylated Tie2 was present only in  $\beta 1$  immune complexes that were isolated from CHO B2a27 (Fig. 2 f). This strengthened the specificity of the Tie2/ $\alpha 5\beta 1$  interaction and demonstrating that it occurred at the plasma membrane.

To verify the *in vivo* association between Tie2 and  $\alpha 5\beta 1$  integrin, we analyzed, by immunofluorescence, the localization of endogenous  $\alpha 5\beta 1$  integrin subunit and Tie2 in ECs plated on fibronectin, and let them adhere for 1 h and 30 min (i.e., until they started developing typical fibrillary contacts). As shown in Fig. S2 (available at <http://www.jcb.org/cgi/content/>

full/jcb.200507082/DC1), most of the endothelial  $\alpha 5\beta 1$  integrin and Tie2 appear to colocalize at the cell membrane in spotty areas that are located outside of typical fibrillary contacts. Linear arrays of Tie2-containing spots also colocalize with some  $\alpha 5\beta 1$  positive fibrillary contacts.

In ECs plated on native ECM, we observed that Tie2 is phosphorylated after 5 min and is active until 1 h of Ang-1 stimulation (unpublished data). To demonstrate that Tie2 sensitization to low Ang-1 concentration was  $\alpha 5\beta 1$ -dependent, we stimulated CHO B2 and CHO B2a27 expressing Tie2 or VEGFR-2 for up to 60 min (Fig. 2, g–l). Ang-1 promoted a higher degree of Tie2 phosphorylation in CHO B2a27 compared with CHO B2 (Fig. 2,

**Figure 2. Tie2 and  $\alpha 5\beta 1$  interact.** ECs cultured on native ECM were lysed and immunoprecipitated by anti- $\alpha 5\beta 1$  (a), anti- $\alpha 2\beta 1$  (b), or anti- $\alpha v\beta 3$  (c) Abs or control immunoglobulins, and blotted with anti-Tie2 (a–c) or anti- $\beta 1$  or anti-VEGFR-2 (a) or anti- $\alpha 2$  (b) or anti- $\beta 3$  (c) Abs. Anti- $\alpha 5\beta 1$  immunoprecipitates were re-immunoprecipitated (Re-IP) by anti-Tie2 Ab or control immunoglobulins and blotted with anti-Tie2 (d). CHO B2 and B2a27 cells expressing Tie2 were lysed, immunoprecipitated with anti- $\beta 1$  Ab, and blotted with anti-Tie2 Ab (e), or biotinylated before lysis, Tie2 or control immunoglobulins re-immunoprecipitated and revealed by peroxidase-conjugated streptavidin (f). CHO B2 and B2a27 cells expressing Tie2 or VEGFR-2 adhered on poly-lysine (CHO B2) or on fibronectin (CHO B2a27) were stimulated with Ang-1 (50 ng/ml) or VEGF-A<sub>165</sub> at the indicated time points; lysed; and immunoprecipitated with anti-Tie2 or anti-VEGFR-2 Abs and blotted with anti-phosphotyrosine (g and i), anti-Tie2 (g) or anti-VEGFR-2 Abs (i). h and i show the densitometric analysis where the Tie2 or VEGFR-2 tyrosine phosphorylation fold increase has been calculated as described in this legend. Values shown are means  $\pm$  SD of five independent experiments. Statistical significance (\*,  $P < 0.01$ ) is shown for Ang-1- or VEGF-A<sub>165</sub>-stimulated cells compared with unstimulated cells.



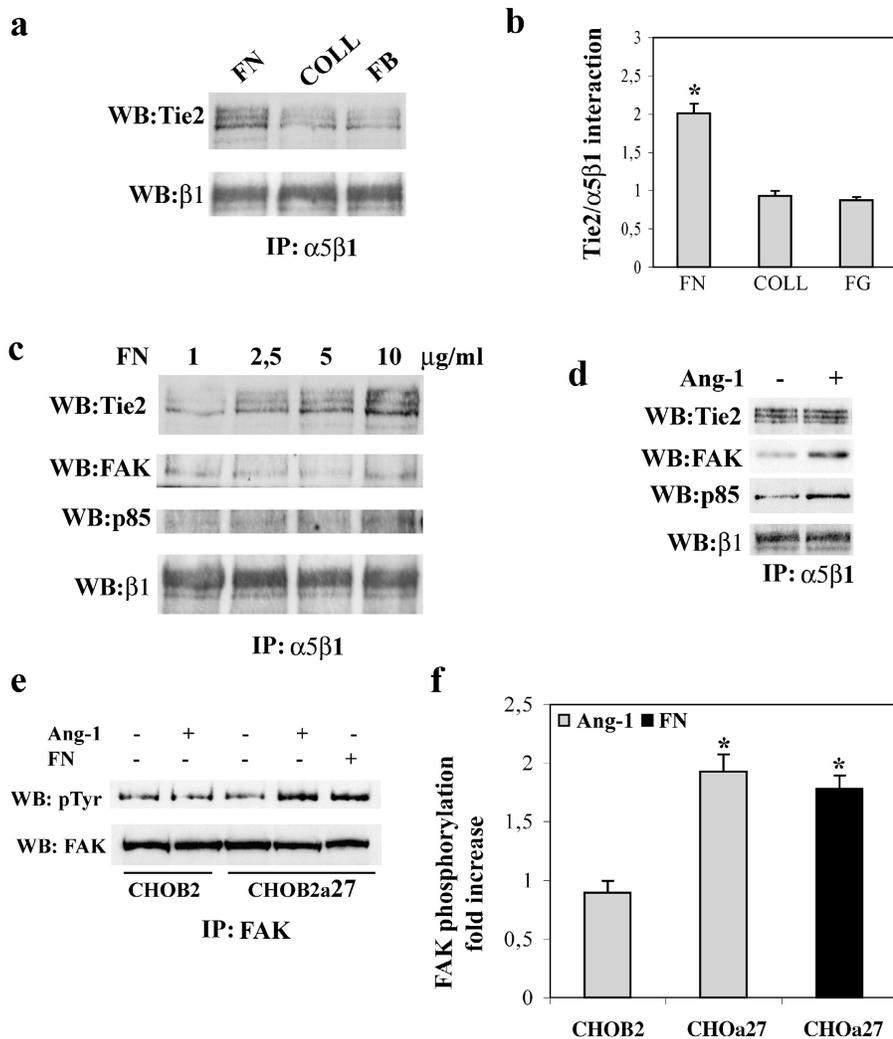
g and h). Furthermore, in CHO cells lacking  $\alpha 5$ , Tie2 phosphorylation declined after 15 min (Fig. 2, g and h). The phosphorylation response of VEGFR-2 to its ligand (Fig. 2, i and j) in the two cell lines did not exhibit the clear-cut differences that were observed for Tie2; this suggested that  $\alpha 5\beta 1$  specifically enhances and prolongs Ang-1-induced Tie2 phosphorylation.

#### The Tie2/ $\alpha 5\beta 1$ complex is induced by fibronectin and activated by Ang-1

Then we investigated the regulation of Tie2/ $\alpha 5\beta 1$  interaction in ECs (Fig. 3). When ECs were plated on fibronectin, the amount of Tie2 coimmunoprecipitated with  $\alpha 5\beta 1$  integrin was remarkably higher than on collagen I or fibrinogen (Fig. 3, a and b). The ability of fibronectin to increase this interaction was dose-dependent (Fig. 3 c), whereas Ang-1 stimulation did not modify the amount of receptor that coimmunoprecipitated with  $\alpha 5\beta 1$  integrin on fibronectin, collagen I, or fibrinogen (Fig. 3 d and data not depicted). To analyze the signaling activity of Tie2/ $\alpha 5\beta 1$  interaction, we assessed the recruitment of the

p85 subunit of PI3-K and FAK to the complex. p85 is an effector of activated Tie2 (Kontos et al., 1998), whereas FAK localizes to integrin clusters and participates in the integrin signaling (Hood and Chersesh, 2002). Tie2 association with fibronectin-activated  $\alpha 5\beta 1$  was not accompanied by the recruitment of FAK and p85 in the  $\alpha 5\beta 1$  immunoprecipitates (Fig. 3 c). EC stimulation by Ang-1 did not modify Tie2 association with  $\alpha 5\beta 1$ , but did increase FAK and p85 recruitment to the complex (Fig. 3 d). Thus,  $\alpha 5\beta 1$  occupancy regulates the stoichiometry of Tie2/ $\alpha 5\beta 1$  association, whereas Ang-1 triggers intracellular signals downstream of Tie2/ $\alpha 5\beta 1$  complexes.

It was shown that coated Ang-1 induces CHO B2a27, but not CHO B2, cell adhesion (Carlson et al., 2001). Therefore, we tested the hypothesis that soluble Ang-1 could promote  $\alpha 5\beta 1$  outside-in signaling in a Tie2-independent way. To exclude integrin engagement, CHO B2 and CHO B2a27 were plated on poly-lysine and then stimulated with soluble Ang-1, which induced a rapid phosphorylation of FAK in CHO B2a27, but not in CHO B2, cells (Fig. 3 e). The increase of FAK phosphorylation



**Figure 3. Tie2/α5β1 complex increases upon α5β1 engagement and transduces upon Ang-1 stimulation.** ECs plated on 5 μg/ml fibronectin, 5 μg/ml collagen I, and 20 μg/ml fibrinogen (a) or on increasing concentrations of fibronectin (1–10 μg/ml; c), or on 5 μg/ml fibronectin (d) were lysed, immunoprecipitated by anti-α5β1 Ab. (d) ECs were stimulated by Ang-1 (50 ng/ml) for 5 min. Blots were probed as indicated. CHO B2 and B2a27 were plated on poly-lysine or fibronectin (e), stimulated or not with Ang-1 (50 ng/ml) for 5 min, lysed, immunoprecipitated with anti-FAK, and blotted with antiphosphotyrosine or anti-FAK. Densitometric analysis shows the relative amount of Tie2 in α5β1 immunoprecipitates expressed in arbitrary unit (b) and the fold increase of FAK phosphorylation (f). Values shown are means ± SD of five independent experiments. Statistical significance (\*,  $P < 0.01$ ) is shown for ECs plated on fibronectin compared with the other conditions, and for Ang-1- or fibronectin-stimulated CHO B2a27 compared with CHO B2.

induced by Ang-1 was similar to that observed when CHOa27 were plated on fibronectin (Fig. 3, e and f). Ang-1-dependent FAK phosphorylation in CHO B2a27 was not blocked by adding anti-α5β1 functional blocking mAb (unpublished data); this suggested that Ang-1- and fibronectin-dependent activation of α5β1 relies upon different molecular mechanisms. These results suggest that Ang-1 could trigger the signaling of the complex through Tie2 and α5β1 downstream effector activation.

#### Ang-1 selectively enhances EC motility on fibronectin

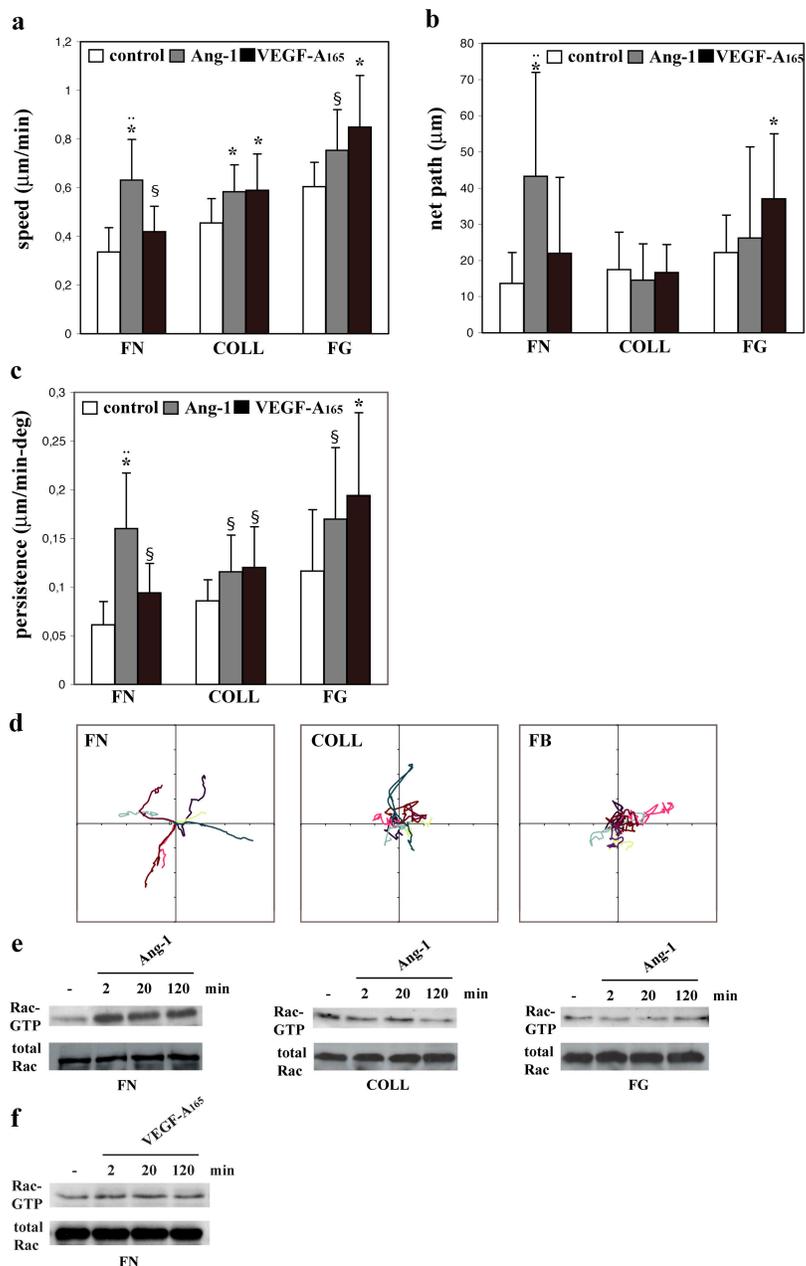
We demonstrated previously that Ang-1 promotes EC chemokinesis by increasing their basal locomotion (Cascone et al., 2003a). We analyzed EC motility on fibronectin, collagen I, and fibrinogen by time-lapse videomicroscopy experiments in the presence or absence of Ang-1. Ang-1 (50 ng/ml) greatly increased the basal speed of ECs plated on fibronectin, whereas its effect was less on cells plated on collagen I and fibrinogen (Fig. 4 a). The net path covered and the migration persistence also were greater on fibronectin (Fig. 4, b and c). In contrast, Ang-1 did not modify the net path covered and had a slight effect on cell persistence on collagen I or fibrinogen (Fig. 4, b and c). The results did not change under increasing Ang-1 concentrations,

independently from the type of substrate (unpublished data). Fig. 4 d shows the tracks of single ECs; this clearly indicates that Ang-1 stimulated a persistent motility of EC on fibronectin, whereas on collagen I and fibrinogen, cells moved randomly around the starting point. As expected (Rousseau et al., 2002; Serini et al., 2003a) VEGF-A<sub>165</sub> was able to increase all chemokinetic parameters studied, but it did not show gains of function on fibronectin compared with the other ECM components (Fig. 4, a–c); this supported the specificity of Ang-1/Tie2 signaling.

Because we demonstrated that Ang-1 induces PI3-K-dependent Rac1-GTP modulation necessary to increase EC basal locomotion (Cascone et al., 2003a), we next looked at the influence of the different substrates on Rac1 activity. Ang-1, but not VEGF-A<sub>165</sub>, triggered a strong and persistent up-regulation of Rac1-GTP in ECs plated to fibronectin. Rac1-GTP peaked at 2 min and persisted for up to 120 min (Fig. 4, e and f). This effect of Ang-1 was negligible in cells plated on collagen I or fibrinogen (Fig. 4 e). Therefore, Ang-1 preferentially promotes directional chemokinesis and Rac1 activation when ECs are engaged by fibronectin.

To support the chemokinetic results further, we studied Ang-1 activity in haptotaxis (Fig. S3 a; available at <http://www.jcb.org/cgi/content/full/jcb.200507082/DC1>). Ang-1 increased

**Figure 4. Ang-1 increases the speed and the persistence of EC plated on fibronectin.** Starved ECs were plated on 5  $\mu\text{g/ml}$  fibronectin (FN), 5  $\mu\text{g/ml}$  collagen I (COLL), and 20  $\mu\text{g/ml}$  fibrinogen (FG) for 1 h and stimulated or not with 50 ng/ml of Ang-1 or 10 ng/ml of VEGF-A<sub>165</sub>. Time-lapse videomicroscopy was performed and recorded images every 5 min for 4 h. 50 cells from five different videos were examined for speed (a), net path length covered (b), and persistence (c; deg, direction). (d) ECs paths were tracked over 4 h and replotted such that all paths start from origin. Values shown are means  $\pm$  SD of 50 cells from five independent experiments. Statistical significance is shown (\*,  $P < 0.01$  and  $\S$ ,  $P < 0.05$ ) for stimulated cells compared with unstimulated cells; \*\*,  $P < 0.01$  for Ang-1-stimulated cells compared with VEGF-A<sub>165</sub>-stimulated cells. ECs plated as in (a) were stimulated with 50 ng/ml of Ang-1 (e) or 10 ng/ml of VEGF-A<sub>165</sub> (f) for the indicated times, and cell lysates were incubated with GST-PBD to pull-down Rac1-GTP. Proteins separated by SDS-PAGE were probed with anti-Rac1 Ab. This experiment is representative of five experiments performed with similar results.

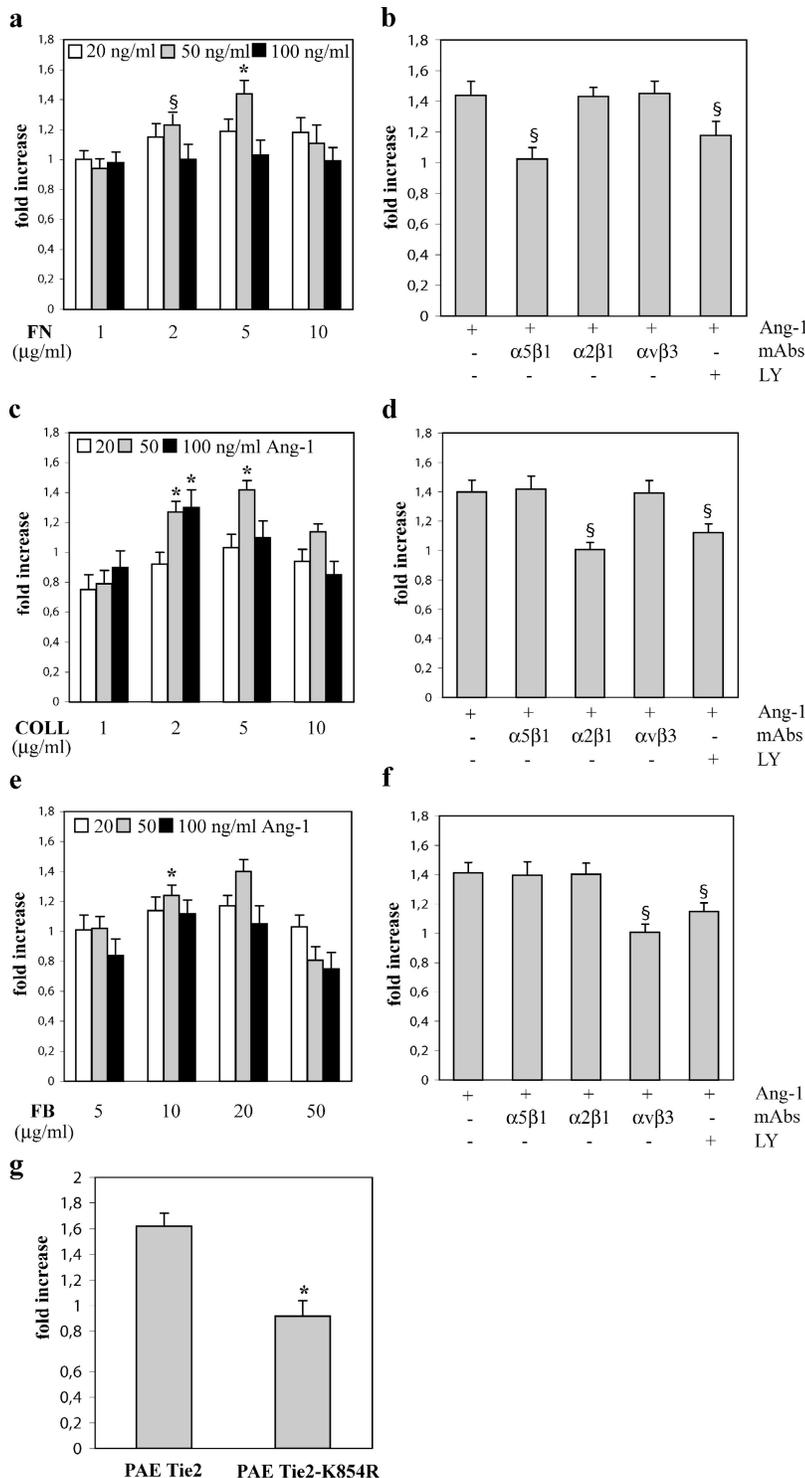


cell migration toward all the substrates tested in a dose-dependent manner. However, the dose-response effect of Ang-1 toward fibronectin was shifted to the left and reached the plateau at 50 ng/ml. Ang-1-dependent haptotaxis activity toward collagen I or fibrinogen was less, appeared at 50 ng/ml, and attained the plateau at 100 ng/ml. VEGF-A<sub>165</sub> did not show any selective effect on EC motility toward fibronectin as compared with collagen I and fibrinogen (Fig. S3 b).

#### Ang-1 enhances integrin-mediated EC adhesion

Growth factor receptors modulate integrin function through PI3-K-dependent inside-out signaling to mediate enhanced biologic effects, such as cell motility, survival, and invasion (Guilherme et al., 1998; Byzova et al., 2000; Baron et al., 2002). Therefore, we verified if the Ang-1-enhanced effect on

fibronectin involved selective  $\alpha 5 \beta 1$  inside-out activation. Then we performed adhesion assays of ECs to fibronectin, collagen I, fibrinogen (Fig. 5), and vitronectin (not depicted) in the presence or absence of Ang-1. Surprisingly, adhesion assays with different concentrations of Ang-1 and ECM proteins showed that Ang-1 increased EC adhesion to a similar extent, independently from the engaged integrins. The highest increase was observed at 50 ng/ml of Ang-1 in ECs adhering on 5  $\mu\text{g/ml}$  of fibronectin or collagen I and 20  $\mu\text{g/ml}$  of fibrinogen (Fig. 5, a, c, and e). Function blocking anti- $\alpha 5 \beta 1$  (Fig. 5 b), anti- $\alpha 2 \beta 1$  (Fig. 5 d), and anti- $\alpha v \beta 3$  (Fig. 5 f) mAbs impaired Ang-1-increased EC adhesion to fibronectin, collagen I, and fibrinogen, respectively; this demonstrated that Ang-1-mediated adhesion was integrin-dependent. Because  $\alpha v \beta 3$  promotes RGD-mediated cell adhesion (Arnaout et al., 2002), anti- $\alpha v \beta 3$  mAb partially inhibited basal EC spreading, but did not block Ang-1-mediated



**Figure 5. Ang-1 induces EC adhesion through specific integrin function.** Panels a, c and e show the fold increase of EC adhesion induced by Ang-1 (20, 50, 100 ng/ml) versus unstimulated ECs to different concentrations of fibronectin (a), collagen I (c), or fibrinogen (e). Values shown are means  $\pm$  SD of five independent experiments, each in triplicate. Statistical significance ( $*$ ,  $P < 0.01$ ,  $^{\S}$ ,  $P < 0.05$ ) is shown for increasing Ang-1 concentrations compared with 20 ng/ml Ang-1. b, d, and f show the fold increase of EC adhesion induced by Ang-1 (50 ng/ml) to 5  $\mu$ g/ml fibronectin (b), 5  $\mu$ g/ml collagen I (d), or 20  $\mu$ g/ml fibrinogen (f) in the presence of function-blocking anti- $\alpha 5\beta 1$  (JBS5), anti- $\alpha v\beta 3$  (LM609), or anti- $\alpha 2\beta 1$  (BHA2.1) Abs, or LY294002. Statistical significance ( $^{\S}$ ,  $P < 0.01$ ) is shown for Abs and LY294002 treatment compared with untreated control. PAE-Tie2 and PAE-Tie2-K854R fold increase adhesion to 5  $\mu$ g/ml fibronectin induced by Ang-1 (50 ng/ml) (g). Values shown are means  $\pm$  SD of five independent experiments. Statistical significance ( $*$ ,  $P < 0.01$ ) is shown for PAE-Tie2-K854R compared with PAE-Tie2.

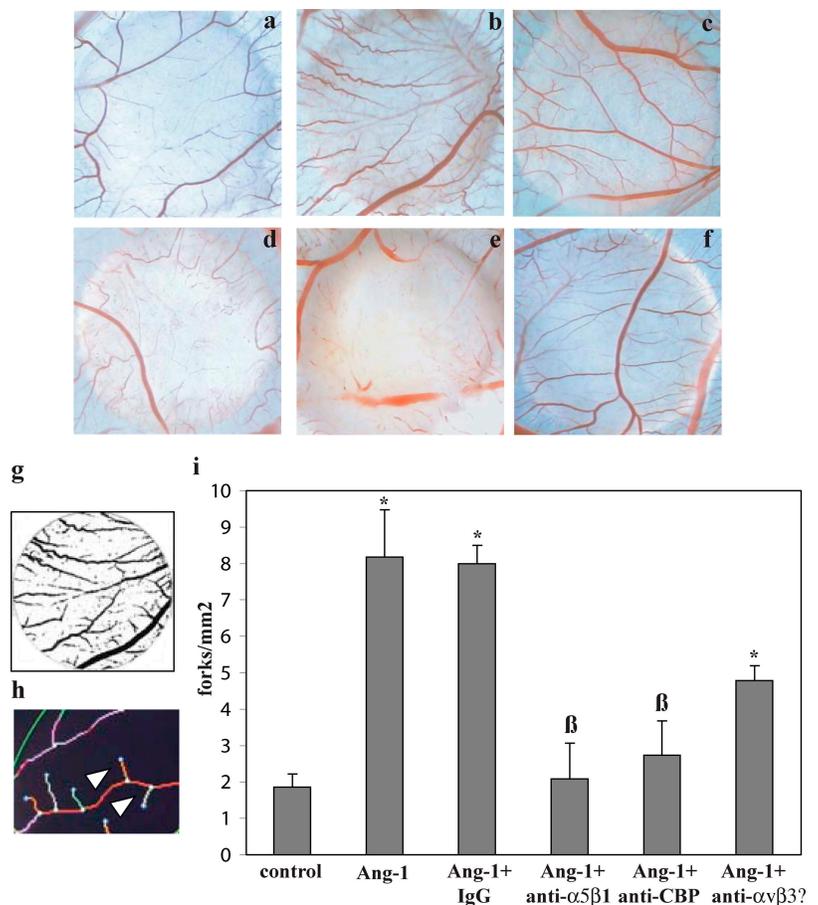
adhesion to fibronectin (Fig. 5 b and not depicted). The PI3-K-specific inhibitor, LY294992, affected Ang-1 proadhesive activity on all ECM ligands (Fig. 5, b, d, and f); this indicated that PI3-K plays a role in this Tie2-dependent inside-out signaling. The role of Tie2 activation in EC adhesion induced by soluble Ang-1 also was investigated in porcine aortic ECs (PAE cells) that were transfected with Tie2 (PAE-Tie2) or with the mutant Tie2-K854R (PAE-Tie2-K854R), which lacks the catalytic activity (Audero et al., 2004; Fig. 5 g). PAE-Tie2-K854R did not

support Ang-1-dependent increased adhesion, which further suggested that this process was Tie2 dependent.

#### Integrin $\alpha 5\beta 1$ is necessary for in vivo Ang-1-induced angiogenesis

Next, we investigated the role of  $\alpha 5\beta 1$  integrin in Ang-1-mediated in vivo angiogenesis. Filter disks saturated with saline or Ang-1 were applied to 10-d-old embryo CAMs. 24 h later, CAMs were treated with function-blocking anti- $\alpha 5\beta 1$  or anti-

**Figure 6.  $\alpha 5\beta 1$  is essential for Ang-1-mediated angiogenesis in CAM assay.** Filter disks saturated with saline (a), Ang-1 (b), Ang-1 treated with control immunoglobulins (c), anti- $\alpha 5\beta 1$  (JBS5; d), anti-CBP (784A2A6; e), and anti- $\alpha v\beta 3$  (LM609; f) Abs. The vessel pattern (g) and forks (h) were analyzed by the imaging software winRHIZO Pro. (i) Measurement of forks/mm<sup>2</sup> by the imaging software winRHIZO Pro. Values shown are means  $\pm$  SD of five independent experiments, each in triplicate. Statistical significance (\*,  $P < 0.01$ ) is shown for Ang-1 treatment compared with control and ( $\beta$ ,  $P < 0.01$ ) for Abs treatment compared with Ang-1 stimulation.



$\alpha v\beta 3$  or anti-fibronectin cell binding peptide mAbs (anti-CBP), which were reported to inhibit growth factor-dependent angiogenesis without affecting preexisting vessels (Brooks et al., 1994; Kim et al., 2000b). 2 d later, CAMs were excised and pictures of filter disks were taken (Fig. 6, a–f; see Supplemental methods). Ang-1 promoted vessel branching and remodeling as compared with saline treatment (Fig. 6, a, b, and i). Anti- $\alpha 5\beta 1$  (Fig. 6 d) and anti-CBP (Fig. 6 e) completely abrogated Ang-1-induced angiogenesis, whereas immunoglobulin treatment was ineffective (Fig. 6, c and i). mAb anti- $\alpha v\beta 3$  (Fig. 6, f and i) only partially inhibited the effect of Ang-1 that maintained a significant angiogenic index versus saline treatment. These results demonstrate that  $\alpha 5\beta 1$  plays a major role in Ang-1-mediated in vivo vascular remodeling and sprouting.

## Discussion

Angiogenesis involves dynamic remodeling of the environment surrounding blood vessels through ECM degradation and deposition of new components. Recent works highlighted the importance of the synergy of signals provided by ECM and growth factors transduced by integrins and specific receptors (Miyamoto et al., 1996; Decker and French-Constant, 2004; Goel et al., 2004). During angiogenesis, integrins are involved in new blood vessel formation as witnessed by studies with integrin antagonists and generation of null mice (Hodivala-Dilke et al., 2003). Moreover, the interaction of  $\alpha v\beta 3$  with VEGFR-2 may mediate

enhanced growth factor-induced cellular response (Soldi et al., 1999; Borges et al., 2000). In this study, we describe the interaction of endothelial receptor Tie2 and  $\alpha 5\beta 1$ , and suggest a new paradigm of angiogenesis modulation that could parallel VEGFR-2/ $\alpha v\beta 3$  cross-talk. The association between Tie2 and  $\alpha 5\beta 1$  is constitutive and regulated through  $\alpha 5\beta 1$  engagement by fibronectin; it results in the sensitization of the receptor to low Ang-1 concentrations and in the acquisition of an efficient directional motility. Furthermore, we demonstrate that  $\alpha 5\beta 1$  activation is necessary in the in vivo angiogenic response to Ang-1.

Integrin occupancy may cause growth factor receptor autophosphorylation (Moro et al., 1998), and several data demonstrate that growth factor receptors and integrins associate under growth factor stimulation (Woodard et al., 1998; Soldi et al., 1999; Lee and Juliano, 2002) or under integrin activation (Benigno et al., 2001; Baron et al., 2002). In ECs, Tie2 is present in free-form and associated with  $\alpha 5\beta 1$  integrin. The activation of  $\alpha 5\beta 1$  integrin by fibronectin increases the complex with Tie2 and modulates the time and concentration window of the receptor activation. When  $\alpha 5\beta 1$  is activated, Tie2 is phosphorylated at low Ang-1 concentrations, whereas at higher Ang-1 concentrations the activation is attenuated. Moreover,  $\alpha 5\beta 1$  expression influences the duration of Ang-1-dependent Tie2 phosphorylation. Therefore, we may speculate that  $\alpha 5\beta 1$  activation could influence Tie2 signal duration and signal strength. It has been reported how the quantitative modulation of signal threshold of tyrosine kinase receptors affects the biologic outcome (Hunter, 2000).

It has been suggested that cooperation between integrins and growth factor receptors may be due to the formation of a complex between FAK and the cytosolic domain of the receptor tyrosine kinase (Sieg et al., 2000). Alternatively, it may involve the direct interaction of the receptors (Borges et al., 2000). Our data show that Tie2 and  $\alpha 5\beta 1$  association occurs independently from cell adhesion and  $\alpha 5\beta 1$  activation. Fibronectin-mediated  $\alpha 5\beta 1$  activation up-regulates the constitutive basal level of Tie2/ $\alpha 5\beta 1$  interaction without increasing FAK in the complex. Therefore, these evidences suggest that Tie2 and  $\alpha 5\beta 1$  complex independently from the presence of a FAK-mediated intracellular bridge, and that this association should be direct, as reported for the interaction between VEGFR-2 and  $\alpha v\beta 3$  (Borges et al., 2000) and further stabilized by conformational changes occurring during integrin activation.

Peculiarly, Ang-1 does not modify the features of association between Tie2 and fibronectin-engaged  $\alpha 5\beta 1$ , but triggers biochemical signals that recruit p85 and FAK to the complex. It is known that p85 binds activated Tie2 (Kontos et al., 1998), whereas FAK is recruited to the cytosolic tail of clusterized integrins at the focal adhesions (Schlaepfer and Mitra, 2004). Thus, Ang-1 stimulation mediates Tie2 and  $\alpha 5\beta 1$  signaling, and allows a cross-talk between these pathways by acting at the level of Tie2/ $\alpha 5\beta 1$  complex. Integrins respond to intracellular cues by modifying the avidity for their ligands. Growth factor receptors modulate integrin function through inside-out signaling to mediate enhanced biologic effects, such as cell motility, survival, and invasion (Byzova et al., 2000; Guilherme et al., 1998; Baron et al., 2002). Here, we demonstrate that Ang-1/Tie2 activates integrins through the PI3-K signaling which suggests that FAK recruitment to Tie2/ $\alpha 5\beta 1$  complex could be dependent on activated Tie2 inside-out signaling. Conversely, the observation that immobilized (Carlson et al., 2001) or soluble Ang-1 binds and activates  $\alpha 5\beta 1$  in the absence of Tie2 demonstrates that Ang-1 promotes  $\alpha 5\beta 1$  outside-in signaling. Therefore, all together the results suggest a model in which the synergism of Tie-2/ $\alpha 5\beta 1$  inside-out/outside-in signaling would allow the stabilization of the complex and the activation of Tie2 at lower Ang-1 concentrations. Furthermore, increased Tie2/ $\alpha 5\beta 1$  interaction on fibronectin promotes selective signaling pathways that modify the chemokinetic response of ECs to Ang-1 with the appearance of a directional motility.

We demonstrated previously that EC chemokinesis mediated by Ang-1 on native ECM is characterized by two peaks of Rac-1 activation that are PI3-K dependent (Cascone et al., 2003b). In this study, we report that Ang-1 induces a prolonged Rac1 activation over a 2-h stimulation in ECs adhered to fibronectin, but not to other ECM molecules. Rac1-GTP mediates lamellipodia extension at the leading edge and participates in growth factor receptors and integrin signaling pathways (Cho and Klemke, 2002; Burrige and Wennerberg, 2004). Furthermore,  $\beta 1$  integrin overexpression increases Rac activity (Miao et al., 2002), and PI3-K/Rac1 loop exerts a positive role in directed motility (Wang et al., 2002); this suggests that Tie2/ $\alpha 5\beta 1$  cross-talk may enhance Ang-1-dependent Rac1 up-regulation, and thus, promote persistent motility.

$\alpha 5\beta 1$  integrin normally is expressed in ECs and is up-regulated during angiogenesis, whereas fibronectin is highly synthesized during embryonic and tissue remodeling and in injured tissues (Armstrong and Armstrong, 2000). Genetic ablation of  $\alpha 5$  or fibronectin leads to embryonic lethality with major vascular defects (George et al., 1993; Yang et al., 1993) and Abs or peptides that block  $\alpha 5\beta 1$  function inhibit growth factor-induced angiogenesis (Kim et al., 2000b). Knock-out mice for Ang-1 or Tie2 show severe defects in vascular remodeling, branching, and maturation (Sato et al., 1995; Suri et al., 1996). Ang-1 has angiogenic effects in many models of postnatal angiogenesis, such as retinal vascularization (Uemura et al., 2002) and hindlimb and myocardial ischemia (Shyu et al., 1998; Chae et al., 2000). In other experimental models, Ang-1 exclusively acts in enhancing VEGF-mediated angiogenic effects (Asahara et al., 1998; Zhu et al., 2002). The role of Ang-1 in tumor angiogenesis seems to be correlated strictly with the tumor model analyzed (Metheny-Barlow and Li, 2003), and is proangiogenic (Shim et al., 2002; Machein et al., 2004) and antiangiogenic (Hawighorst et al., 2002; Stoeltzing et al., 2003). In this study, we show that Ang-1 induces vessel remodeling in CAM assay, and that this process depends on  $\alpha 5\beta 1$  integrin and the cell-binding domain of fibronectin, but not on  $\alpha v\beta 3$  integrin. Interestingly, in the same in vivo model, antagonists of  $\alpha 5\beta 1$  integrin blocked bFGF-, TNF- $\alpha$ -, and IL-8-stimulated angiogenesis, but had a minimal effect on VEGF-A response (Kim et al., 2000b). These evidences indicate that Ang-1 elicits angiogenesis through a strict cooperation with activated  $\alpha 5\beta 1$  integrin. Functional cross-talk between Tie2 and  $\alpha 5\beta 1$  is highlighted by similarities in the defects of heart development in mice that were genetically modified for  $\alpha 5$  and Ang-1 or Tie2 (Yang et al., 1993; Dumont et al., 1994; Suri et al., 1996). Expression of  $\alpha 5\beta 1$  in ECs correlates with angiogenesis, and is engaged in ECM remodeling sites where fibronectin and other ECM components accumulate to mediate dynamic interactions with activated ECs (Kalluri, 2003). Recently, Thurston et al. (2005) described how Ang-1 promotes the enlargement of vessels in a critical window of vascular plasticity in neonatal mice. Therefore, we point out that Ang-1/Tie2 biologic effects could be regulated by microenvironment changes that characterize the angiogenic switch occurring in tissues. In conclusion, we demonstrate that Tie2 and  $\alpha 5\beta 1$  structurally and functionally cross-talk in vitro and in vivo, and lead to a fine-tuning modulation of the vascular effect of Ang-1.

## Materials and methods

### Cell culture and reagents

Human umbilical vein ECs were cultured and used as described previously (Bussolino et al., 1992). PAE cells (provided by J. Waltenberger, University Medical Center, Ulm, Germany) constitutively expressing human Tie-2 or human Tie2-K854R were cultured as described (Audero et al., 2004). CHO clone B2 cell line expressing <2% of endogenous hamster  $\alpha 5\beta 1$  and CHO clone B2/ $\alpha 27$  expressing pECE- $\alpha 5$  were provided by R. Juliano (University of North Carolina, Chapel Hill, NC; Bauer et al., 1993). In some experiments, CHO B2 and B2/ $\alpha 27$  were transfected with pCDNA3-Tie2, or pCDNA3-VEGFR-2 provided by Georg Breier (Max Planck Institute for Physiological and Clinical Research, Bad Nauheim, Germany).

Cells were cultured on wells or transwells (Costar) or dishes (Falcon) coated with fibronectin, collagen I, or fibrinogen at the indicated concen-

trations, and saturated with 3% BSA. Human recombinant Ang-1 and VEGF-A<sub>165</sub> were from R&D Systems.

For the antibodies used, see online supplemental materials (available at <http://www.jcb.org/cgi/content/full/jcb.200507082/DC1>).

#### Adhesion assay

ECs and PAE cells were starved in 0.5% FCS. 100 cells/ $\mu$ l were plated in the presence of Ang-1 on 96-well microtiter plates, incubated for 30 min at 37°C, washed three times with PBS, fixed in 3.7% glutaraldehyde, and stained with 0.1% crystal violet. In some experiments, JBS5 anti- $\alpha$ 5 $\beta$ 1 (2  $\mu$ g/ml), LM609 anti- $\alpha$ v $\beta$ 3 (2  $\mu$ g/ml), BHA2.1 anti- $\alpha$ 2 $\beta$ 1 (5  $\mu$ g/ml) Abs (Chemicon), or LY294002 (15  $\mu$ M) were added to cell suspension. This drug concentration did not modify cell viability as assessed by trypan blue exclusion. The absorbance was read at 540 nm in a spectrophotometer for microtiter plate (HT6 7000 Bio Assay Reader, PerkinElmer).

#### Motility assay

Starved ECs were plated onto 20-mm dishes and allowed to adhere in 0.5% FCS for 1 h at 37°C. Ang-1 or VEGF-A<sub>165</sub> was added to the medium, and ECs were observed with an inverted microscope equipped with thermostatic and CO<sub>2</sub> controlled chamber (model DM IRB HC; Leica). Phase-contrast video images of control and stimulated ECs were recorded at 5-min intervals for 4 h using a CCD camera (Hamamatsu Photonics), and were analyzed for velocities of cell migration, path length covered, and directionality of cell motility using DIAS image processing software (Solltech) as described previously (Serini et al., 2003b). Speed, net path length, and persistence parameters of 50 cells from five experiments were calculated and plotted.

#### Immunoprecipitation

Cells were washed with cold PBS and lysed in buffer with added protease and phosphatase inhibitors (50  $\mu$ g/ml pepstatin, 50  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100  $\mu$ M ZnCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>). To immunoprecipitate integrin complexes, a buffer containing 50 mM HEPES (pH 7.4), 5 mM EDTA, 2 mM EGTA, 150 mM NaCl, 10% glycerol, and 1% NP-40 was used. For all of the other conditions of immunoprecipitation, a buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol was used. In some experiments, cells were labeled for detection of cell surface proteins (ECL Protein Biotinylation Module, Amersham Biosciences), washed three times with ice-cold PBS, and incubated with bicarbonate buffer containing biotinamidocaproate N-hydroxysuccinamide ester for 30 min at 4°C before lysis. Lysates (1 mg) were precleared with nonimmune goat serum or mouse IgG and protein G-Sepharose (Amersham Biosciences) for 2 h at 4°C and then incubated with protein G-Sepharose or protein A-Sepharose and Abs indicated for 2 h at 4°C. In re-immunoprecipitation experiments, the immune complexes from anti- $\alpha$ 5 $\beta$ 1 immunoprecipitation were resuspended in 50 mM Tris-HCl pH 7.4, 2% SDS and heated for 5 min at 95°C. Supernatants were diluted 10-fold with lysis buffer and immunoprecipitated with anti-Tie2 Ab. After washes, immunoprecipitates were resolved on SDS-PAGE, immunoblotted as indicated or probed with peroxidase-conjugated streptavidin, and detected by the enhanced chemiluminescence technique (PerkinElmer). Densitometry represent the results of at least five independent experiments.

#### Chick CAM angiogenesis assays

For CAM assay, we produced some changes to the previously described method (Valdembri et al., 2002). Fertilized chick embryos were incubated for 3 d at 37°C at 70% humidity. A small hole was made over the air sac at the end of the egg and a second hole was made directly over the embryonic blood vessels. After 10 d, cortisone acetate-treated filter disks (5 mm) saturated with 50–200 ng of Ang-1 or saline were placed on the CAM in an area with a minimum of small blood vessels. The day after, anti- $\alpha$ 5 $\beta$ 1 (JBS5; 15  $\mu$ g), anti- $\alpha$ v $\beta$ 3 (LM609; 10  $\mu$ g), or anti (anti-CBD; 784A2A6; 25  $\mu$ g) function-blocking Abs or IgG (25  $\mu$ g) was applied on the filter disks, and eggs were incubated for 2 d. The concentrations were chosen based on previously published experiments (Kim et al., 2000b).

#### CAM analysis

CAMs were fixed with PBS-3.7% paraformaldehyde for 10 min at room temperature, filter disks were excised, and pictures were taken with a JVC TK-C1380E color video camera (ImageProPlus 4.0 imaging software) connected to the stereomicroscope (model SZX9; Olympus). Pictures were processed with the imaging software winRHIZO Pro (Regent Instruments Inc.). This software reproduces vessel pattern (Fig. 6 g), identifies vessel branching, and gives back the forks (blood vessel branch points) pro area

(arrows in Fig. 6 h). Data represent the results of five independent experiments, each performed in triplicate.

#### Statistical analysis

Statistical analysis was performed using the unpaired *t* test or one-way analysis of variance and Bonferroni's test for pairwise multiple comparisons (SPSS 13.0; SPSS Inc.).

#### Online supplemental material

Fig. S1 shows EC survival data. Fig. S2 shows the colocalization of Tie2 and  $\alpha$ 5 $\beta$ 1. Fig. S3 shows haptotaxis data. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200507082/DC1>.

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## Supplemental materials and methods

### Antibodies

The anti- $\alpha$ 5 $\beta$ 1 (AB1950), anti- $\alpha$ 2 $\beta$ 1 (clone BHA2.1), anti- $\alpha$ 2 $\beta$ 1 (JBS2), anti- $\alpha$ v $\beta$ 3 (25E11), anti- $\alpha$ 5 $\beta$ 1 (clone JBS5), anti-fibronectin cell binding peptide (anti-CBP) (clone 784A2A6), and the anti- $\alpha$ v $\beta$ 3 (clone LM609) antibodies were from CHEMICON International; the anti- $\alpha$ 2, anti- $\beta$ 1A, and anti- $\alpha$ 5 antibodies were a gift of Dr. Guido Tarone (University of Torino, Torino, Italy); the anti- $\beta$ 3 (SC-6627), anti-VEGFR-2 (SC-6251) and anti-Tie2 (SC-324) antibodies were from Santa Cruz Biotechnology, Inc.; the anti-p85 (06-195) antibody and anti-phosphotyrosine (05-321) monoclonal antibodies (mAbs) were from Upstate Biotechnology; the anti-FAK (clone 77) and anti-Rac mAbs were from Becton Dickinson; and the anti-VEGFR-2 (55B11) was from Cell Signaling Technology.

### Survival assay

$2.5 \times 10^4$  ECs were plated on 48-well plates and were allowed to adhere in 20% FCS medium. ECs were then starved for 1 h in 0.5% FCS medium and treated with Ang-1 or VEGF-A<sub>165</sub> at the indicated concentrations. 24 h later, cells were washed with PBS and incubated in phenol red-free DMEM containing 0.2 mg/ml MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium)] for 2 h (Kwak et al., 2000). The absorbance was read at 595 nm.

### Immunofluorescence

For all experiments, coverslips were coated for 1 h at 37°C (5  $\mu$ g/ml fibronectin), blocked for 30 min at 37°C (3% BSA). Cells were allowed to adhere, were fixed with 3.7% PFA, permeabilized with 0.1% Triton X-100 for 2 min, blocked with 3% BSA for 15 min at 37°C, and stained with mouse monoclonal anti-Tie2 (AB33; Cell Signaling Technology) (1:50) for 30 min at 37°C and with TRITC-conjugated anti-mouse IgG (AlexaFluor 555; Molecular Probes, Invitrogen) for 45 min at RT. Coverslips were then stained with anti- $\alpha$ 5 integrin antibody (1:50) followed by FITC-conjugated anti-rabbit IgG (AlexaFluor 488; Molecular Probes, Invitrogen).

### Haptotaxis

For haptotaxis experiments, 24-well transwell chambers (Costar) containing polycarbonate filters with 8- $\mu$ m pores were used. The underside of the filters were coated with fibronectin (5  $\mu$ g/ml), collagen type I (5  $\mu$ g/ml), or fibrinogen (20  $\mu$ g/ml), as indicated in the Materials and methods section. Starved ECs were resuspended in M199 0.5% FCS at the concentration of 280 cells/ $\mu$ l, in presence of Ang-1 or VEGF-A<sub>165</sub> at the indicated concentrations and of cell suspension was seeded in the upper chamber, and M199 0.5% FCS was added to the lower chamber. Cells were incubated for 3 h at 37°C in a 95% air and 5% CO<sub>2</sub> atmosphere. After washing with PBS, migrated cells to the bottom side of the transwell membrane were fixed with 3.7% glutaraldehyde and cells on the upper side of the filter were carefully removed by scraping with the edge of a cotton swab. ECs were stained with crystal violet, and the absorbance was read at 540 nm.

### GTPases assay

Rac1-GTP pull-down was performed as previously described (Cascone et al., 2003a). Starved ECs were plated as described in the Immunoprecipitation section of Materials and methods, were stimulated with Ang-1 or VEGF-A<sub>165</sub>, lysed, and incubated with 30  $\mu$ g GST-PBD fusion molecules bound to Glutathione-Sepharose beads (GE Healthcare). After washes, the solubilized proteins were resolved on 12% SDS-PAGE and immunoblotted with anti-Rac1 mAb.

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