Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2

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The Tie1 receptor tyrosine kinase was isolated over a decade ago, but so far no ligand has been found to activate this receptor. Here, we have examined the potential of angiopoietins, ligands for the related Tie2 receptor, to mediate Tie1 activation. We show that a soluble Ang1 chimeric protein, COMP-Ang1, stimulates Tie1 phosphorylation in endothelial cells with similar kinetics and angiopoietin dose dependence when compared with Tie2. The phosphorylation of overexpressed Tie1 was weakly induced by COMP-Ang1 also in transfected cells that do not express Tie2. When cotransfected, Tie2 formed heteromeric complexes with Tie1, enhanced Tie1 activation, and induced phosphorylation of a kinase-inactive Tie1 in a ligand-dependent manner. Tie1 phosphorylation was also induced by native Ang1 and Ang4, although less efficiently than with COMP-Ang1. In conclusion, we show that Tie1 phosphorylation is induced by multiple angiopoietin proteins and that the activation is amplified via Tie2. These results should be important in dissecting the signal transduction pathways and biological functions of Tie1.

Introduction

Tie1, an endothelial-specific receptor tyrosine kinase, shares a high degree of homology with Tie2, the receptor for the angiopoietins (Yancopoulos et al., 2000; Jones et al., 2001; Peters et al., 2004). The expression of the *Tie1* gene is restricted to endothelial cells and to some hematopoietic cell lineages (Partanen et al., 1992; Korhonen et al., 1994; Dumont et al., 1995; Hashiyama et al., 1996; Yano et al., 1997). Up-regulation of Tie1 expression has been observed during wound healing, ovarian follicle maturation, and tumor angiogenesis (Korhonen et al., 1992; Kaipainen et al., 1994).

Targeted disruption of the mouse *Tie1* gene results in embryonic lethality after embryonic day (E) 13.5 because of severe edema, hemorrhages, and defective microvessel integrity (Puri et al., 1995; Sato et al., 1995). Tie1 is required cell autonomously for endothelial cell survival and extension of the

vascular network during late embryogenesis, particularly in regions undergoing angiogenic growth of capillaries (Puri et al., 1995; Sato et al., 1995). Tie2 knockout mouse embryos die by E10.5 due to endocardial defects, hemorrhaging, and impaired vascular network formation (Dumont et al., 1994; Sato et al., 1995). Embryos lacking both Tie1 and Tie2 revealed an absolute requirement for Tie2 in the endocardium at E10.5, whereas both receptors were dispensable for the initial assembly of the rest of the vasculature (Puri et al., 1999). In contrast, both receptors were required in the microvasculature during late organogenesis, in essentially all blood vessels of the adult and for postnatal bone marrow hematopoiesis (Puri and Bernstein, 2003).

Although Tie1 is an orphan receptor with no known ligands, three members of the angiopoietin family (Ang1, Ang2, and Ang3/4) have been identified as ligands for Tie2 (Lee et al., 2004; Peters et al., 2004). Ang1 promotes vascular remodeling, maturation, and stabilization of the vasculature, and the Ang1 null phenotype is very similar to the Tie2 null phenotype resulting in embryonic lethality at E12.5 (Suri et al., 1996). Overexpression of Ang2 in the blood vessels mimicked the phenotype of Tie2 null embryos and led to lethality at E9.5–10.5 (Maisonpierre et al., 1997), suggesting an antagonist function for Ang2 in Tie2 signaling. In endothelial cells, Ang1

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Abbreviations used in this paper: BEC, blood vascular endothelial cell; DTSSP, 3,3′-dithiobis[sulfosuccinimidylpropionate]; E, embryonic day; HUVEC, human umbilical vein endothelial cell; LEC, lymphatic endothelial cell.
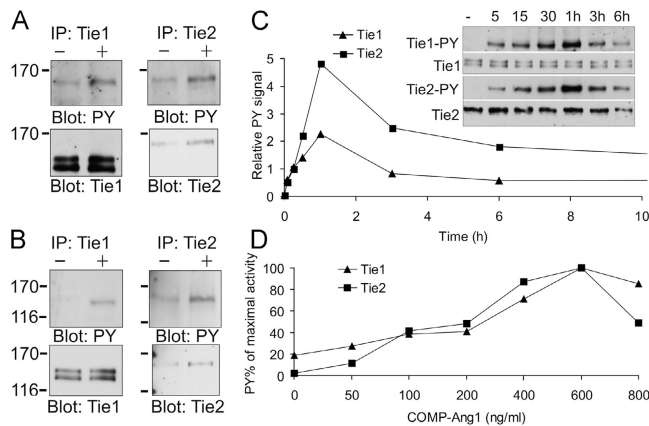
Figure 1. **COMP-Ang1 induces tyrosine phosphorylation of Tie1 in endothelial cells.** (A and B) Isolated BECs (A) and LECs (B) were starved overnight and stimulated with COMP-Ang1 for 15 min or left unstimulated. The cell lysates were immunoprecipitated with anti-Tie1 or anti-Tie2 antibodies. Aliquots of the immunoprecipitates were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine, anti-Tie1, or anti-Tie2 antibodies. Relative mobilities of the molecular mass markers are indicated in kilodaltons. (C) HMEC-1 cells were stimulated with COMP-Ang1 for the indicated times followed by analysis of Tie1 and Tie2 as explained in A. The chemiluminescence signal was quantitated using Fluorchem digital imaging system (Flowgen Bioscience). Shown is relative phosphotyrosine intensity normalized to Tie1 or Tie2 protein levels. (D) HMEC-1 cells were stimulated with indicated amounts of COMP-Ang1 for 30 min followed by analysis of Tie1 and Tie2 as in C. The results are shown as a percentage of maximal receptor phosphorylation.

Figure 2. **Soluble Tie2 receptor inhibits COMP-Ang1–induced Tie1 phosphorylation.** (A) EA.hy926 cells were stimulated with COMP-Ang1 or Ang2 or left unstimulated, followed by immunoprecipitation and analysis of Tie1 and Tie2, as in Fig. 1. White line indicates that intervening lanes have been spliced out. (B) EA.hy926 cells were stimulated with COMP-Ang1 alone or in combination with 10 μg/ml of soluble Tie1-Fc or Tie2-Fc for 15 min. As a control, cells were treated with soluble Tie receptors only. Tie1 and Tie2 were analyzed as in Fig. 1.

induced Tie2 phosphorylation, whereas Ang2 did not (Maisonpierre et al., 1997), unless the cells were treated for a prolonged period (Teichert-Kuliszewska et al., 2001). To date, none of the angiopoietins have been shown to induce Tie1 activation.
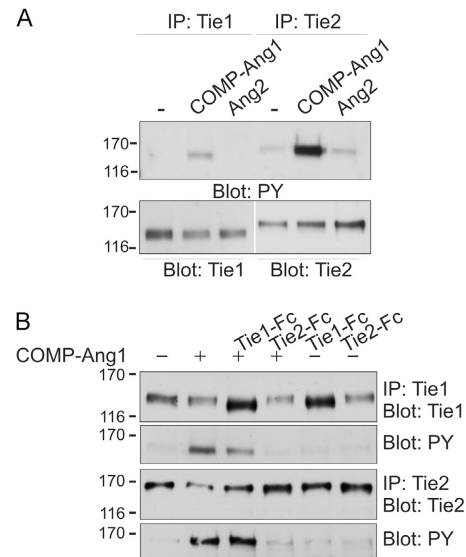
Without a ligand, the signal transduction pathway downstream of Tie1 activation and responsible for its biological function has been difficult to elucidate. However, experiments using a chimeric receptor composed of the extracellular domain of colony-stimulating factor-1 receptor and the intracellular domain of Tie1 showed that activated Tie1 associates with the phosphatidylinositol 3-kinase, resulting in activation of the serine/threonine kinase Akt (Kontos et al., 2002). This result protected cells from apoptosis, indicating overlapping functions of Tie1 and Tie2 (Kontos et al., 1998).

Here, we have reassessed the possibility that the angiopoietins may affect Tie1 function. We show that a chimeric Ang1 protein, native Ang1, and Ang4 induced Tie1 phosphorylation in primary and immortalized endothelial cells. Chimeric Ang1 induced weak phosphorylation of Tie1 overexpressed in nonendothelial cells, and this activation was enhanced by Tie2 coexpression. Furthermore, we demonstrate heteromeric Tie1–Tie2 complexes in cells expressing both receptors. Our results suggest that Ang1 and Ang4 proteins can activate Tie1 and that Tie2 significantly amplifies the activation.

Results and discussion

COMP-Ang1 induces tyrosine phosphorylation of Tie1 in endothelial cells

To investigate the signal transduction pathways of Tie1, we stimulated human dermal blood vascular endothelial cells

(BEC) and lymphatic endothelial cells (LEC; Makinen et al., 2001) with the COMP-Ang1 chimeric protein (Cho et al., 2004a,b). Surprisingly, COMP-Ang1 induced tyrosine phosphorylation of Tie1, in addition to phosphorylation of Tie2 (Fig. 1, A and B). Phosphorylation of Tie1 occurred in endothelial cells within 5 min of COMP-Ang1 stimulation, reaching a maximum level at 1 h, followed by a gradual down-regulation (Fig. 1 C). The kinetics of Tie2 phosphorylation paralleled these changes. Significant phosphorylation occurred with 100 ng/ml COMP-Ang1, but maximal phosphorylation of both receptors required 600 ng/ml (Fig. 1 D).

COMP-Ang1 induced phosphorylation of Tie1 and Tie2 also in the hybrid endothelial cell line EA.hy926 (Fig. 2 A). In contrast, 600 ng/ml Ang2 did not activate either Tie1 or Tie2. In fact, decreased Tie1 phosphorylation was seen when COMP-Ang1 was provided in combination with an excess of Ang2 (unpublished data).

The soluble extracellular domain of Tie2 (Tie2-Fc) has been found to bind Ang1 and to inhibit Ang1 induced Tie2 activation, whereas no effect has been found with the soluble Tie1 receptor (Davis et al., 1996; Peters et al., 2004). Tie2-Fc inhibited COMP-Ang1–induced Tie1 and Tie2 phosphorylation (Fig. 2 B), whereas Tie1-Fc had little if any effect, indicating that COMP-Ang1 binds to the soluble form of Tie2 but not to soluble Tie1, although COMP-Ang1 was capable of inducing activation of Tie1 at the cell surface.

COMP-Ang1 induces Tie1 phosphorylation, which is enhanced by Tie2

To understand the mechanism of COMP-Ang1–induced Tie1 activation, we overexpressed Tie1 in 293T cells, which lack both Tie1 and Tie2 (Fig. 3 A). Variable and low levels of Tie1 tyro-
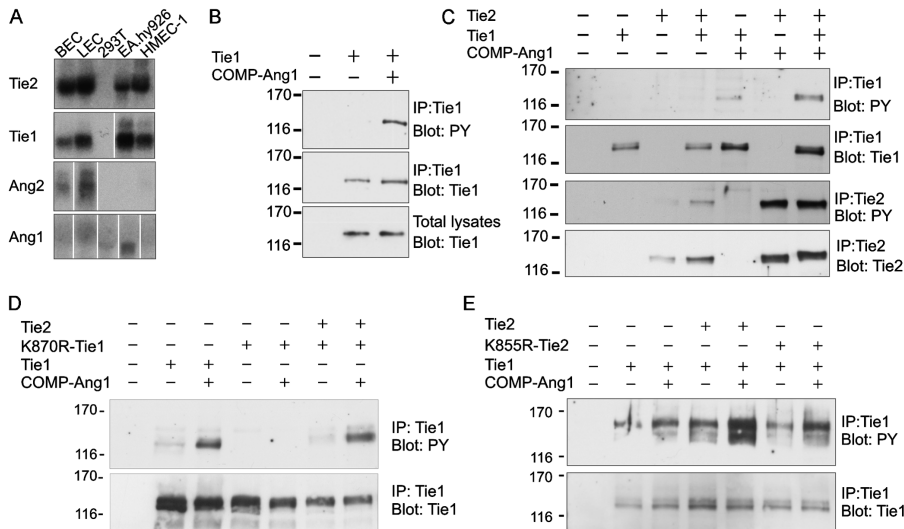
Figure 3. **COMP-Ang1–induced Tie1 phosphorylation is enhanced by Tie2.** (A) The expression of human Ang1, Ang2, Tie1, and Tie2 was analyzed using Northern blotting of total RNA from the cell lines indicated. White lines indicate that intervening lanes have been spliced out. (B) 293T cells were transfected with Tie1 expression vector, as indicated, and stimulated or not with COMP-Ang1. Tie1 was analyzed as in Fig. 1. (C) 293 cells were transfected with expression plasmids for Tie1 and Tie2, as indicated, followed by analysis of Tie1 and Tie2 as in Fig. 1. (D) 293T cells were transfected with expression plasmids for wild-type or kinase-inactive Tie1 (K870R-Tie1) and Tie2, as indicated, followed by analysis of Tie1 as in Fig. 1. (E) 293 cells were transfected with expression plasmids for Tie1 and wild-type or kinase-inactive Tie2 (K855R-Tie2), followed by analysis of Tie1 as in Fig. 1.

sine phosphorylation were detected after stimulation of these cells with 600 ng/ml COMP-Ang1 (Fig. 3 B). This finding suggested that overexpressed Tie1 can be activated to some degree by high concentrations of COMP-Ang1 in the absence of Tie2.

Next, we examined the effect of Tie2 on Tie1 activation by COMP-Ang1 in the transfected cells. Because of the strong basal autophosphorylation of Tie2 in 293T cells (unpublished data), we used 293 cells that do not replicate transiently transfected expression plasmids. 293 cells were transfected with vectors encoding Tie1, Tie2, or both and stimulated with COMP-Ang1. COMP-Ang1–induced tyrosine phosphorylation of Tie1 was increased in the double transfected cells in comparison with cells transfected only with Tie1, suggesting that heteromerization of Tie1 and Tie2 enhances Tie1 activation (Fig. 3 C). In contrast, Tie2 phosphorylation was not enhanced by the presence of Tie1 when compared with cells transfected with Tie2 alone.

It was possible that Tie2 was required for high-affinity binding of COMP-Ang1 to Tie1 or that Tie2 induced the phosphorylation and thereby enhanced the activation of Tie1 in a Tie1–Tie2 complex. To analyze this hypothesis, we expressed K870R-Tie1 that has an inactivating substitution in the kinase domain with or without Tie2 (Fig. 3 D). K870R-Tie1 was phosphorylated in a ligand-dependent manner when coexpressed with Tie2, whereas no phosphorylation was detected in the absence of Tie2. Thus, Tie2 was able to induce Tie1 phosphorylation.

We also tested if a kinase-inactive K855R-Tie2, like wild-type Tie2, was able to enhance Tie1 phosphorylation. However, we found that Tie1 phosphorylation was reduced when it was coexpressed with K855R-Tie2 (Fig. 3 E), indicating that the kinase activity of Tie2 is required for full enhancement of Tie1 activation by COMP-Ang1.

Tie1 forms a heteromeric complex with Tie2

The results obtained from the transfected cells suggested that Tie1 and Tie2 undergo heteromerization when stimulated by COMP-Ang1. To analyze this finding, we used 293T cells transfected with Tie1-V5 and Tie2-Myc constructs. After COMP-Ang1 stimulation, the cell surface proteins were chemically cross-linked with 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP), a membrane nonpermeable cross-linker, and Tie1 was immunoprecipitated from the cell lysates. Interestingly, Tie2 was coprecipitated with Tie1 from the double transfected cells (Fig. 4 A). The treatment of human umbilical vein endothelial cells (HUVECs) with DTSSP resulted in coprecipitation of Tie1 with Tie2, whereas no coprecipitation was found in nontreated cells (Fig. 4 B). In conclusion, Tie1 and Tie2 form heteromeric complexes on the cell surface.

In line with these results, Marron et al. (2000) have reported interaction between the intracellular domains of the two Tie receptors using the TrkA ectodomain fused to the Tie1 or Tie2 endodomain. However, until now there was no evidence that angiopoietins could induce Tie1 activation or heteromeric Tie1–Tie2 complexes. Our results also suggest that in the heteromeric complexes Tie2 directly phosphorylates Tie1, as Tie2 induced phosphorylation of kinase-inactive Tie1 in a COMP-Ang1–dependent manner.

COMP-Ang1 has been shown to be a more potent angiopoietin ligand than native Ang1 (Cho et al., 2004a,b). We analyzed if also native Ang1 can induce Tie1 phosphorylation.
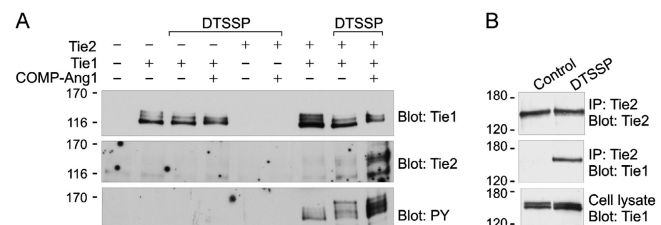
Figure 4. **Tie1 and Tie2 interact at the cell surface.** (A) 293T cells were transfected with Tie1-V5 or Tie2-Myc expression plasmids, as indicated. The cells were stimulated with COMP-Ang1 or left unstimulated, followed by chemical cross-linking of the cell surface proteins with DTSSP. Tie1 was immunoprecipitated using anti-V5 antibodies and analyzed as in Fig.1. (B) HUVECs were treated with control vehicle or DTSSP, followed by Tie2 immunoprecipitation. The immunoprecipitates and cell lysates were separated in SDS-PAGE and immunoblotted with anti-Tie2 and anti-Tie1 antibodies.

Figure 5. **Native Ang1 and Ang4, but not Ang2 or Ang3, induce Tie1 phosphorylation.** (A and B) EA.hy926 cells were stimulated with indicated amounts of COMP-Ang1, native Ang1, Ang2, Ang3, Ang4, or COMP-HFARP or left unstimulated, followed by immunoprecipitation and analysis as in Fig. 1.
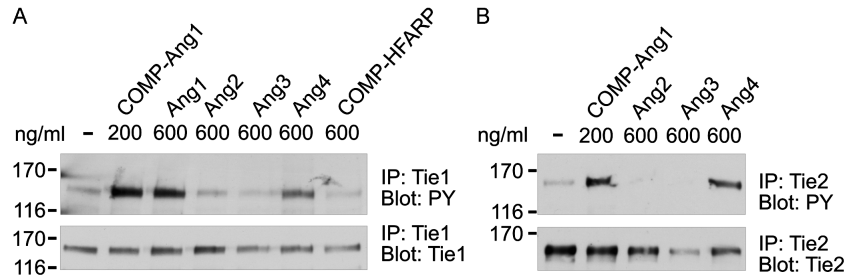
As shown in Fig. 5 A, native Ang1 induced Tie1 phosphorylation in endothelial cells, although severalfold less efficiently than COMP-Ang1. In contrast, COMP-HFARP (hepatic fibrinogen/angiopoietin-related protein) that does not bind to Tie1 or Tie2 (Kim et al., 2000) had no effect even at high concentrations. Thus, COMP-Ang1–induced Tie1 activation is mediated via Ang1 and not by the COMP domain. In addition to Ang1, Ang4 provides a ligand for human Tie2, whereas Ang3 is a specific ligand for murine Tie2 (Lee et al., 2004). We found that Tie1 phosphorylation was induced by native Ang4 but not by Ang3 or Ang2 (Fig. 5 A).

We show here that Tie1 is activated in cells stimulated by COMP-Ang1, a highly soluble and active form of human Ang1 that binds to Tie2 and forms mainly pentamers as compared with trimers, tetramers, and high order multimers formed by native Ang1 (Cho et al., 2004a,b). Tie1 phosphorylation has also been detected in BOW-Ang1-treated rat endothelial cells (Papadopoulos, N., and G. Thurston, personal communication). Because of the poor solubility of the multimeric Ang1 growth factor, the vast majority of published work has been conducted with an Ang2/Ang1 chimeric protein called Ang1* (Koblizek et al., 1998). Like Ang1*, COMP-Ang1 has an intact COOH-terminal receptor binding domain, but includes a coiled coil domain distinct from the multimerization and coiled coil domains that occupy the NH_2 terminus of native Ang1 (Cho et al., 2004a,b). However, despite the lower specific activity of native angiopoietin ligands, we found that Ang1 and Ang4 can also activate Tie1; likewise these ligands are known to activate human Tie2 (Lee et al., 2004).

Variable, low levels of Tie1 phosphorylation were induced by COMP-Ang1 also in cells overexpressing Tie1 but lacking Tie2. This finding was surprising as Ang1, Ang1*, or COMP-Ang1 do not interact with the soluble Tie1-Fc receptor (Davis et al., 1996; Cho et al., 2004a; Peters et al., 2004). In line with these results, Tie1-Fc was unable to inhibit COMP-Ang1–induced Tie1 phosphorylation (our present data). It is possible that the overexpressed Tie1 has a very weak affinity to the Ang1 receptor-binding domain and that membrane association of Tie1 is required to enhance its binding to Tie1, as shown for ligand binding to erbB2 (Tzahar et al., 1997). Ang1 could also induce a conformational change in Tie1, thereby enhancing its affinity for Ang1, which could not be mimicked by the soluble Tie1 extracellular domain. Alternatively, COMP-Ang1–induced Tie1 activation could involve complex formation with additional molecules, such as integrins, with which Ang1 has been shown to interact (Carlson et al., 2001). Although the details of how Ang1 and

Ang4 interact with Tie1 remains to be worked out, these angiopoietins can clearly activate the Tie1 receptor. These data are consistent with studies showing that the phenotype of the Tie1/Tie2 double knockout mice is more severe than that of the Tie1 or Tie2 single knockouts (Partanen and Dumont, 1999; Peters et al., 2004). Our results on Ang1-induced Tie1 activation now call for a better understanding of the complex biological signaling of Ang1 via the two Tie receptors in angiogenesis, lymphangiogenesis, and hematopoiesis.

Materials and methods

Reagents and cell culture
293, 293T (American Type Culture Collection), and EA.hy926 immortalized hybrid HUVECs (Edgell et al., 1983) were grown in DME supplemented with 10% FBS (PromoCell). HUVECs were cultured as described previously (Marron et al., 2000). LEC, BEC (Makinen et al., 2001), and HMEC-1 human dermal microvascular cells immortalized with SV40 Large T antigen (Ades et al., 1992) were grown in Endothelial Cell Basal Medium (PromoCell) with supplements provided by the manufacturer. Confluent plates of cells were serum-starved overnight, followed by ligand stimulation for 15 min, unless otherwise indicated. The following reagents were used: Tie1-Fc, Tie2-Fc, Ang1, VEGF (all from R&D Systems), Ang2, Ang3, Ang4 (Lee et al., 2004), COMP-HFARP (Kim et al., 2000), and Ang2 (Scharpfenecker et al., 2005). The following antibodies were used: antiphosphotyrosine (4G10; Upstate Biotechnology), anti-Tie1 and anti-Tie2 (R&D Systems; Santa Cruz Biotechnology, Inc.; clone 33 [Upstate Biotechnology]), anti-V5 (Invitrogen), and anti-Tie2 (Harris et al., 2001).

DNA constructs and transfection
Cells were transfected using Fugene6 (Roche Diagnostics), changed to serum-free medium after 48 h, and harvested 72 h after transfection. Kinase-inactivating mutation in human Tie2 (lysine 855 to arginine), human Tie1 (lysine 870 to arginine), Tie1-V5, and Tie2-Myc constructs were created by PCR. All constructs were confirmed by sequencing (Applied Biosystems).

Immunoprecipitation and immunoblotting
Cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 5% glycerol, 1 mM EGTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF, 1 mM Na_3VO_4, PMSF, aprotinin, and leupeptin) or alternatively in SDS-lysis buffer (Saharinen et al., 1997). Equal amounts of cell lysate protein were precleared by incubation with protein G–Sepharose (Amersham Biosciences), followed by addition of BSA (1%) and specific antibodies. The immunocomplexes, captured by protein G–Sepharose, were separated in 7.5% SDS-PAGE (Ready-Gels; Bio-Rad Laboratories) and blotted and detected using specific primary antibodies, biotinylated anti–mouse or anti–goat secondary antibodies (DakoCytomation), and streptavidin-biotin HRP conjugate (Amersham Biosciences) followed by ECL detection with the SuperSignal West Femto Maximun Sensitivity Substrate (Pierce Chemical Co.).

HUVECs were cross-linked in PBS containing 0.5 mM DTSSP for 30 min, quenched by addition of Tris, pH 7.5, to 100 mM, and lysed in 50 mM Tris, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA, and complete protease inhibitor. 293T cells were cross-linked for 40 min with 1 mM DTSSP on ice.

RNA isolation and Northern blotting
Total RNA was isolated using the RNeasy kit (QIAGEN), electrophoresed, blotted, and hybridized with ^{32}P-labeled cDNA probes.

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