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Adaptor ShcA Protein Binds Tyrosine Kinase Tie2 Receptor and Regulates Migration and Sprouting but Not Survival of Endothelial Cells*

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Enrica Audero‡§, Ilaria Cascone‡§, Fabrizio Maniero‡§, Lucia Napione‡§, Marco Arese‡§, Luisa Lanfrancone¶, and Federico Bussolino‡§‖

From the ‡Division of Molecular Angiogenesis, Institute for Cancer Research and Treatment (IRCC), School of Medicine, University of Torino, 10060 Candiolo, Italy, the §Department of Oncological Sciences, University of Torino, 10060 Candiolo, Italy, and the ¶European Institute of Oncology, 20141 Milano, Italy

Angiopoietin-1 can promote migration, sprouting, and survival of endothelial cells through activation of different signaling pathways triggered by the Tie2 tyrosine kinase receptor. ShcA adapter proteins are targets of activated tyrosine kinases and are implicated in the transmission of activation signals to the Ras/mitogen-activated protein kinase pathway. Here we report the identification of an interaction between the adapter protein ShcA and the cytoplasmic domain of Tie2 through *in vitro* co-immunoprecipitation analysis. Stimulation of endogenous Tie2 in endothelial cells with its ligand angiopoietin-1 increased its association with ShcA and phosphorylation of the adapter protein. The interaction requires the SH2 domain of ShcA and the tyrosine phosphorylation of Tie2 as shown by pull-down experiments. Furthermore, Tyr-1101 of Tie2 was identified as the primary binding site for the SH2 domain of ShcA. Overexpression of a dominant-negative form of ShcA affects angiopoietin-1-induced chemotaxis and sprouting, although it has no effect on survival of endothelial cells. Furthermore, this mutant partially reduces the tyrosine phosphorylation of the regulatory p85 subunit of phosphatidylinositol 3-kinase. Together, our results identified a novel interaction between Tie2 with the adapter molecule ShcA and suggested that this interaction may play a role in the regulation of migration and three-dimensional organization of endothelial cells induced by angiopoietin-1.

The organization and remodeling of blood vessels is a tightly regulated process controlled in part by paracrine signals, many of which are initiated by the binding of growth factor ligands to their cognate transmembrane receptor tyrosine kinases (RTKs)[1] expressed on the surface of endothelial cells (ECs) (1, 2). The endothelium-specific receptor tyrosine kinase Tie2 is thought to be an important modulator of both normal physiologic and pathologic angiogenesis (3). Tie2 activity is regulated by two naturally occurring secreted ligands. Angiopoietin (Ang)-1 induces Tie2 phosphorylation and promotes migration, sprouting, and survival of ECs *in vitro* (4–7). A second ligand, Ang-2, has a tissue-specific modulatory effect on Tie2 activity. Ang-2 blocks the ability of Ang-1 to activate Tie2 in ECs, but it activates Tie2 expressed in hemangioblast precursor cells or ectopically expressed in fibroblasts (8, 9).

Signal transduction pathways triggered by Tie2 have been extensively examined. Tyr-1101of Tie2 directly associates in a phosphotyrosine (pTyr)-dependent manner with the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, which in turn activate PI 3-kinase, leading to cell motility and survival (10–12). Tie2 also binds Dok-R, an adapter molecule structurally homologous to $p62^{dok}$ and insulin-receptor substrate-3 (13). Dok-R is able to recruit the adapter protein Nck and Ras-GTPase-activating protein. Upstream molecules of the Ras/mitogen-activated protein kinase pathway (MAPK) pathway, Grb2 and Shp2, were also identified as binding partners of Tie2 (11, 14). Finally, signal transducers and activators of transcription factor-3 and -5 are activated by Tie2 in association with the increased expression of p21 cell cycle inhibitor (15).

The SH2-containing ShcA proteins are cytoplasmic substrates of RTKs and have been implicated in the transmission of activation signals to Ras proteins (16, 17). Three *Shc* genes have been identified in mammals, and their gene products have been referred to as ShcA, ShcB, and ShcC (18, 19). ShcA is ubiquitously expressed, whereas ShcB and ShcC expression appear limited to neuronal cells (18, 20). ShcA is expressed as three overlapping polypeptides of 46, 52, and 66 kDa that are produced through alternative splicing and differential use of translation initiation sites and differ only in the length of their amino-terminal extensions (16, 21). Shc proteins possess at their carboxyl terminus an SH2 domain, and toward the amino terminus, these proteins possess a phosphotyrosine binding (PTB) domain. Following RTK association, Shc proteins become phosphorylated at the tyrosine residues 239, 240, and 317 (22, 23). Both Tyr-239 and Tyr-240 form an optimal binding site for the SH2 domain of the adapter Grb2. The Ras guanine nucleotide exchange factor Sos is recruited to this complex through

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‖ To whom correspondence should be addressed: Division of Molecular Angiogenesis, Institute for Cancer Research and Treatment (I.R.C.C.), Sp. 142, Km 3.95, 10060 Candiolo, Torino, Italy. Tel.: 11-9933347; Fax: 11-9933524; E-mail: federico.bussolino@ircc.it.

[1] The abbreviations used are: RTKs, transmembrane receptor tyrosine kinases; Ab: antibody; Ang, angiopoietin; EC, endothelial cell; mAb, monoclonal Ab; MAPK, mitogen-activated protein kinase; SH2 or SH3, Src homology 3; PI, phosphatidylinositol; PTB, phosphotyrosine binding; pTyr, phosphotyrosine; FCS, fetal calf serum; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

the association with the SH3 domain of Grb2, providing a mechanism through which Shc phosphorylation can lead to activation of the Ras-MAPK pathway (24, 25). Shc can also associate, either directly or via Grb2, with the docking protein Gab1 that in turn binds PI 3-kinase (26). Thus, ShcA appears to be a scaffold for the assembly of signaling proteins involved in the activation of the Ras-MAPK pathway and potentially other signaling pathways.

It has been recently shown that ShcA is specifically expressed in the developing cardiovascular system during early embryogenesis (27). Targeted disruption of mouse *shcA* leads to selective defects in vessel organization into a mature vascular network, although the principal vascular progenitors are properly assembled. This is most evident in yolk sac and head vasculature in which vessels are dilated and remain relatively uniform in size. Thus, embryos deficient for ShcA have defects in later stages of vascular patterning that are very similar to those observed in embryos deficient for the Tie2 receptor and its ligand, Ang-1 (28, 29). These data suggest that ShcA may be a target for the Tie2 receptor and may participate in the transduction of the Ang-1 signal in ECs.

In this report, we demonstrate that the adapter molecule ShcA is recruited and activated by the Tie2 receptor upon Ang-1 stimulation in ECs. Overexpression of a dominant-negative form of Shc affects Ang-1-induced migration of sprouting but not survival of ECs. These results suggest the potential role of ShcA in mediating the signaling pathways triggered by the Tie2 receptor that is selectively involved in migration and three-dimensional organization of ECs.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Antibodies—All of the reagents unless specified were purchased from Sigma. ECs from human umbilical veins were prepared and characterized as described previously (30). They were used at early passages (I–IV). COS7 cells (HTB-14, ATCC, Manassas, VA) and Phoenix Ampho cells (kindly provided by G. Nolan, Stanford University) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Sigma).

Rabbit polyclonal antibody (Ab) against human Tie2 and the mouse monoclonal Ab (mAb) against and human c-Myc were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-pTyr mAb and anti-Vav and anti-p85 Abs were purchased from Upstate Biotechnology Inc (Lexington, NY). Polyclonal and monoclonal anti-human ShcA were described previously (16, 31, 32).

Production of recombinant Ang-1 was achieved using a baculovirus expression system as described previously (33), and the amount of protein in the supernatant was estimated by comparison with bovine serum albumin standards quantified by densitometric analysis (Phoretix 1D, Nonlinear USA Inc, Durham, NC). As control, conditioned medium from Sf9 cells infected with wild-type baculovirus (mock) was used.

Immunoprecipitation and Immunoblotting—Confluent ECs (1×10^7 cells/15-cm dish) were made quiescent by 16 h of starvation in M199 containing 0.5% FCS and 0.1% bovine serum albumin and then stimulated for the indicated period of time at 37 °C. Total protein extracts were obtained by lysing cells with boiling extraction buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 10% glycerol). For immunoprecipitation experiments, cells were lysed in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and protease and phosphatase inhibitors (50 μg/ml pepstatin, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 μM $ZnCl_2$, 1 mM Na_3VO_4). After centrifugation (20 min, 10,000 $\times$ *g*), supernatants were incubated with protein A-Sepharose (Amersham Biosciences) and anti-Tie2 Ab (1:50) or anti-p85 Ab (1 μg/ml) or with protein G-Sepharose (Amersham Biosciences) and mAb anti-Shc (1:100) for 2 h at 4 °C. Immunoprecipitates were washed four times with lysis buffer, and proteins were solubilized under reducing conditions, separated by SDS-PAGE (10%), transferred to a nitrocellulose membrane (Amersham Biosciences), and probed with the indicated Abs. The enhanced chemiluminescence technique (PerkinElmer Life Sciences) was used for detection. Protein concentration was determined using the BCA protein assay system (Pierce).

Isolation of Tie2 cDNA and Site-directed Mutagenesis—Tie2 full-length cDNA was cloned from ECs by reverse transcriptase-PCR. The primers 5′-GTTTTTGAAAGGATCCTTGGGACCTCAT-3′ and 5′-CCAAGCAGTGAGATCTTCACTCCA-3′ were used to amplify the cDNA portion corresponding to the extracellular domain of Tie2 (amino acids 1–631). The primers 5′-GGAGTGAAGATCTCACTGCTTGG-3′ and 5′-GCAGAGGCATGAATTCTCAGCAGT-3′ were used to amplify the cDNA portion corresponding to the transmembrane and cytoplasmic domains (amino acids 632–1125). The two cDNA fragments were reconstructed into the pCDNA3.1 vector (Invitrogen). To generate the carboxyl-terminal mutations, an *in vitro* oligonucleotide site-directed mutagenesis system (Promega, Madison, WI) was used. Tie2 cDNAs carrying the appropriate Tyr-Phe mutations were reconstructed in the pCDNA3.1 vector for transient expression in COS7 cells using the DNA-calcium phosphate co-precipitation procedure (Invitrogen).

Pull-down Experiments—GST-ShcSH2 fusion proteins corresponding to ShcA-SH2domain domains was cloned, expressed, and purified exactly as described previously (31). GST fusion proteins (15 μg) were coupled to GSH-Sepharose beads (Amersham Biosciences). Lysates from COS7 cells (2×10^6 confluent cells) transfected with wild-type or mutant Tie2 cDNAs were incubated with the immobilized GSTs for 90 min at 4 °C in the presence of 1 mM Na_3VO_4. Beads were washed three times with lysis buffer, and proteins were solubilized in boiling Laemmli buffer, separated by SDS-PAGE (10%), and immunoblotted with an anti-Tie2 Ab.

Retroviral Infection and Immunofluorescence Analysis—SH2 Vav domain (from amino acid 670 to 721) cDNA was cloned from Jurkat T cells by reverse transcriptase-PCR. The primers 5′-TGGTGCGGCAGAGGGTG-3′ (position 2011–2027 from the ATG) and 3′-GGGGAACTGCAAGGTGGTG-5′ (position 2291–2267) were used to amplify the SH2-cDNA portion corresponding to amino acids 670–721. The cDNAs of ShcASH2 (32) and VavSH2 domains were Myc-tagged by recombinant polymerase chain reaction in pPCR2.1 vector (Invitrogen) and subsequently subcloned into EcoRI-EcoRI sites of the retroviral vector PINCO (34, 35). The two transgene domains and green fluorescence protein (GFP) cDNA were under the control of the long terminal repeat and cytomegalovirus promoters, respectively. Amphotropic cell line Phoenix was transfected with PINCO, PINCO-ShcSH2, and PINCO-VavSH2 to produce retroviral supernatant with high viral titer. The virus-containing medium was collected, filtered (0.45-μm filter, Millipore), and supplemented with 4 μg/ml Polybrene. ECs at first passage were plated onto 150-mm tissue culture dishes, and culture medium was replaced with the viral supernatant. Cells were incubated for 5 h at 37 °C, and then fresh growing medium was added. After 72 h of infection, cells were analyzed for GFP expression both under fluorescence microscope and through fluorescence-activated cell sorting analysis (FACS Advantage S. E., BD Biosciences). Immunofluorescence analysis with anti-c-Myc Ab was performed as described previously (35).

Migration Assay—Migration assay was performed as described previously with a modified Boyden chamber technique (30). Polycarbonate filters (5-μm pore size polyvinylpyrrolidone-free, Neuroprobe, Pleasanton, CA) were coated with 0.1% gelatin for 30 min at room temperature. Recombinant Ang-1 in M199 supplemented with 2% bovine serum albumin and 2% FCS was added to the lower compartment of the chamber, and 50 μl of cell suspension (1.5×10^6 cells/ml) in M199 containing 2% bovine serum albumin and 2% FCS were then seeded in the upper compartment. Checkerboard analysis of the chemotactic response was performed by varying the concentrations of Ang-1 in the upper and lower compartment of the Boyden Chamber (30). After 5 h of incubation, filters were removed and cells were fixed in methanol, stained with Giemsa solution (Diff-Quick, Baxter Diagnostics, Rome, Italy), and counted from five random high power fields (magnification $\times$100) in each well. Each experimental point was studied in triplicate.

Sprouting Assay—ECs were suspended at a density of 4,000 cells/ml in culture medium containing 20% Methocel stock (12 mg/ml carboxymethylcellulose in M199) and 20% FCS (36). 800 cells were seeded into non-adherent round bottom 96-well plates (BD Falcon) and cultured overnight at 37 °C. The spheroids were harvested by gently pipetting and centrifuged at 300 $\times$ *g* for 15 min. The spheroids were then resuspended in 200 μl of M199 medium containing 40% FCS, 1.2% (v/w) methylcellulose, and the indicated amount of Ang-1. An equal volume of a collagen solution (7 volumes of collagen from rat tail, 1 volume of 10$\times$ M199, 1 volume of 0.1 N NaOH, 1 volume of 0.2 M Hepes, pH 7.3) was added. Spheroids were then seeded in 96-well plates and incubated at 37 °C. After 24 h, phase-contrast images were captured by using a CCD Hamamatsu ORCA (Hamamatsu Photonics Italia, Arese, Italy) camera linked to an inverted microscope (model DM IRB HC; Leica Microsystems, Heerbrugg, Switzerland).

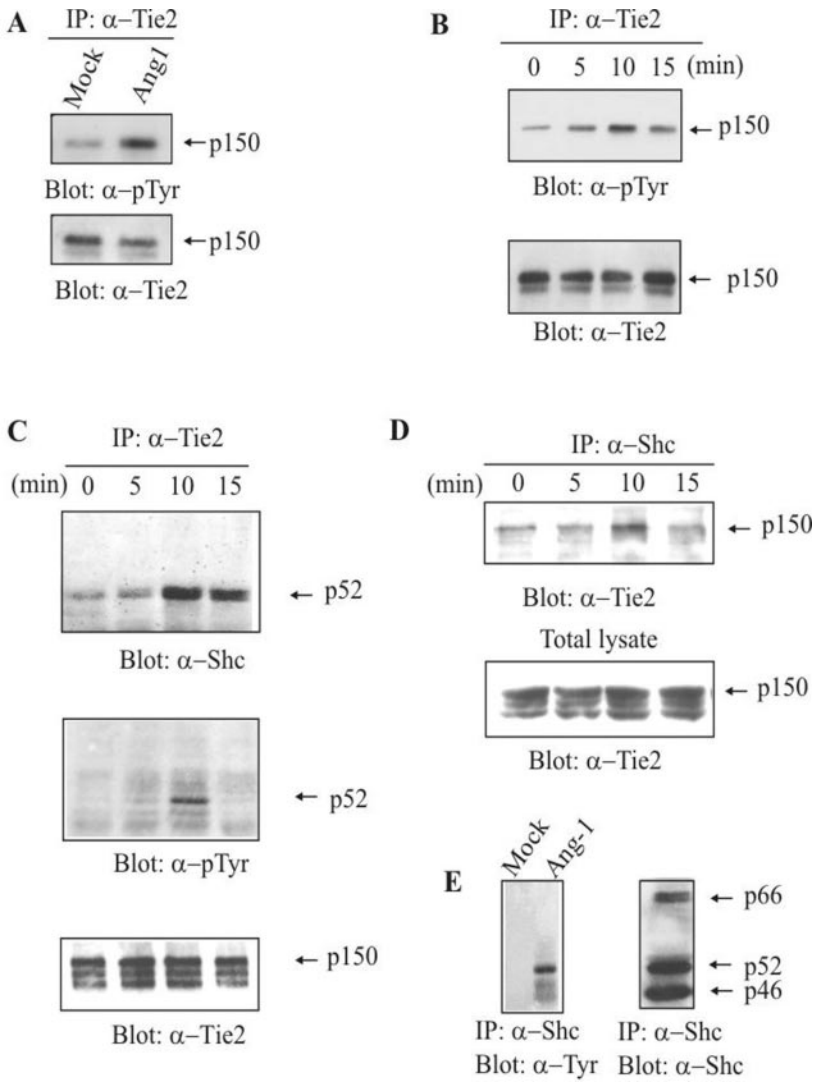

FIG. 1. **Tyrosine-phosphorylated form of Sch binds to Tie2 activated by Ang-1.** *A*, starved ECs were incubated with mock or Ang-1 (200 ng/ml) for 10 min at 37 °C. Cell lysates were subjected to immunoprecipitation (*IP*) with anti-Tie2 Ab and then immunoblotted (*Blot*) with an anti-pTyr mAb (*top panel*). Immunoblot was stripped and reprobed with anti-Tie2 Ab (*bottom panel*). *B*, starved ECs were stimulated with Ang-1 (200 ng/ml) for the indicated period of time at 37 °C. Cell lysates were subjected to immunoprecipitation with anti-Tie2 Ab and immunoblotted with an anti-pTyr mAb (*top panel*). The immunoblot was stripped and reprobed with anti-Tie2 Ab. (*bottom panel*). *C*, starved ECs were stimulated with Ang-1 (200 ng/ml) for the indicated period of time. Cell lysates were subjected to immunoprecipitation with anti-Tie2 Ab and immunoblotted with an anti-ShcA Ab (*top panel*). The immunoblot was stripped and reprobed with a mAb anti-pTyr (*middle panel*) or anti-Tie2 (*bottom panel*). *D*, ECs were serum-starved and incubated with Ang-1 (200 ng/ml) for the indicated period of time. Cell lysates were immunoprecipitated with anti-ShcA Ab and immunoblotted with anti-Tie2 Ab (*top panel*). Equivalent amounts (50 μg) of the same protein lysate were subjected to immunoblot analyses with anti-Tie2 Ab (*bottom panel*). *E*, starved ECs were stimulated for 10 min with Ang-1 (200 ng/ml) at 37 °C, and the cell lysate was immunoprecipitated by anti-Sch Ab and immunoblotted with an anti-pTyr mAb (*left panel*). In *right panel*, ECs were immunoprecipitated by anti-Sch Ab and immunoblotted with Ab anti-Sch. The panels here shown are representative of at least four experiments.

MTT Assay—ECs were plated on collagen-coated 24-well plates and allowed to adhere overnight, and then the medium was removed and the cells were washed once with phosphate-buffered saline. Fresh medium containing 0.5% FCS was added, and the cells were starved for 6 h. After starvation, Ang-1 (200 ng/ml) or vascular endothelial growth factor-A_{165} (20 ng/ml, R&D System, Minneapolis, MN) was added to the cells maintained in M199 containing 0.5% FCS. Cells were incubated at 37 °C for 12, 24, and 48 h. Fresh medium with growth factors was added every 24 h. Cells were then washed in phosphate-buffered saline and incubated with 0.2 mg/ml MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) in Dulbecco's modified Eagle's medium without phenol red for 2 h. The formazan product derived from the reduction of the yellow MTT tetrazolium salt by mitochondrial reductases was dissolved in dimethyl sulfoxide for 5 min. Formazan absorbance was assessed at 595 nm in a Bio Assay Reader HTS 7000 (PerkinElmer Life Sciences) (36, 37).

RESULTS

ShcA Binds Tie2 Receptor and Is Tyrosine-phosphorylated upon Ang-1 Stimulation—To examine the signaling properties of Tie2, recombinant Ang-1 was produced in Sf9 insect cell using a baculovirus vector. Ang-1 expressed with this system was functionally active and able to induce Tyr phosphorylation of Tie2, whereas the mock supernatant was ineffective (Fig. 1*A*). The optimal dose of Ang-1 that activated Tie2 was 200 ng/ml as previously determined (data not shown and Ref. 33) and was routinely used in the biochemical experiments reported here. A time course experiment performed challenging ECs with this concentration of Ang-1 showed that Tie2 phosphorylation increased as early as 5 min, reached a maximum at 10 min, and then declined (Fig. 1*B*).

Phosphorylation of Shc proteins is an early event in intracellular signaling in a number of well characterized RTKs (17). The ability of Ang-1-activated Tie2 to bind and phosphorylate ShcA proteins was investigated in ECs. Serum-starved ECs were incubated with Ang-1 for the indicated period at 37 °C. Tie2 was then immunoprecipitated from the cell lysates, and proteins were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-ShcA Ab (Fig. 1*C*, *top panel*). Association between Tie2 and p52 ShcA was easily detectable at 10 min of stimulation and decreased after 15 min. No association between Tie2 and the p46 and p66 ShcA proteins could be detected, although the cells expressed high amounts of ShcA isoforms (Fig. 1*E*, *right panel*). The phosphorylation state of ShcA from control and Ang-1-treated cells was evaluated by Western blotting using an anti-pTyr mAb. As illustrated in Fig. 1*C* (*middle panel*), phosphorylation in Tyr residues of p52 Sch associated with Tie2 was evident after 10 min of stimulation with Ang-1 and then drastically reduced after 15 min. In the reverse experiment, lysates of unstimulated and Ang-1-stimulated ECs were incubated with anti-ShcA Ab and the precipitated proteins were immunoblotted with an anti-Tie2 Ab. The amount of the receptor co-immunoprecipitated with ShcA increased after 10 min of stimulation with Ang-1 (Fig. 1*D*). Furthermore, immunoprecipitated p52Sch was phosphorylated in

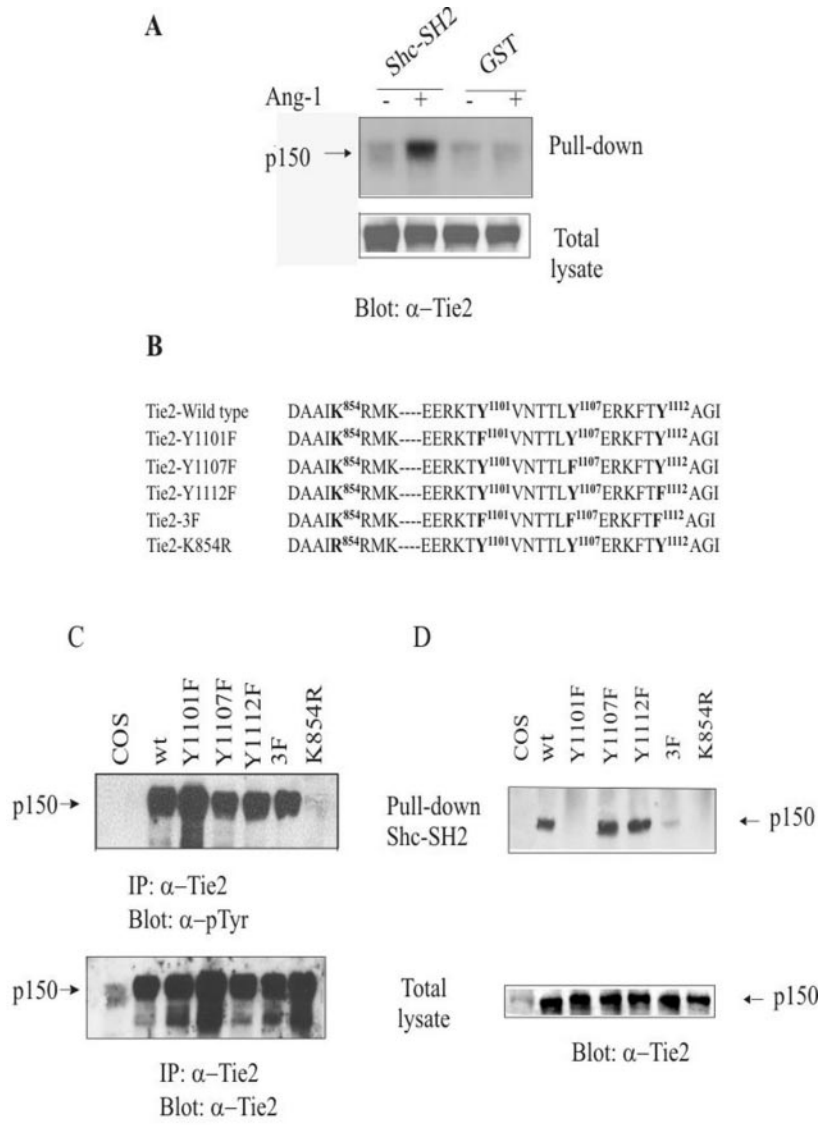

FIG. 2. **Identification of putative docking sites for ShcA on Tie2 receptor.** *A*, GST fusion proteins of SH2 domain of ShcA or GST alone were immobilized on GSH-Sepharose and incubated with lysates from unstimulated and Ang-1-stimulated COS7 cells (one confluent 10-cm dish) transiently transfected with Tie2 cDNA. Proteins were resolved by SDS-PAGE and immunoblotted with anti-Tie2 Ab. 50 μg of the same protein lysates were subjected to immunoblot analyses with anti-Tie2 Ab (*bottom panel*). *B*, amino acid sequence of Tie2 receptor. In *boldface* are indicated the residues mutated for every Tie2 mutants. *C*, wild-type (*wt*) and mutant Tie2 cDNAs were transiently transfected in COS7 cells that were stimulated with Ang-1 (200 ng/ml for 10 min). Lysates were immunoprecipitated (*IP*) with anti-Tie2 Ab and immunoblotted (*Blot*) with mAb anti-pTyr (*top panel*) or anti-Tie2 (*bottom panel*). *D*, GST fusion protein of the SH2 domain of ShcA was immobilized on GSH-Sepharose and incubated with lysates from Ang-1-stimulated COS7 cells (200 ng/ml for 10 min) transiently transfected with wild-type and mutant Tie2 cDNAs. Proteins were resolved by SDS-PAGE and immunoblotted with anti-Tie2 Ab. Equivalent amounts (50 μg) of the same protein lysate were subjected to immunoblot analyses with anti-Tie2 Ab (*bottom panel*). The panels here shown are representative of four experiments.

Tyr residues in EC stimulated for 10 min by Ang-1 (Fig. 1*E*). Taken together, these results indicate that Tie2 activation upon Ang-1 stimulation induces p52 ShcA association with the receptor and its Tyr phosphorylation.

Identification of Tie2 Tyrosine Phosphorylation Sites Required for Interaction with SH2 Domain of ShcA—Shc proteins participate in tyrosine kinase receptor-mediated cell activation mainly through the carboxyl-terminal SH2 domain that recognizes specific pTyr residues. To investigate whether the SH2 domains of ShcA could directly bind the Tie2 receptor, an *in vitro* binding experiment was performed using GST-fused Sch-SH2 protein immobilized on GSH-Sepharose beads. COS7 cells were transiently transfected with Tie2 cDNA and challenged with Ang-1. The lysates were incubated with the immobilized GSTs and immunoblotted with anti-Tie2 Ab (Fig. 2*A*). SH2 domain was able to bind Tie2 in response to Ang-1 stimulation, whereas the GST alone did not precipitate the receptor.

The carboxyl-terminal tail of Tie2 contains three tyrosine residues (Tyr-1101, Tyr-1107, and Tyr-1112) through which the receptor seems to trigger several cascades of molecular protein-protein interactions (3). Another Tyr residue (Tyr-815) is present in the juxtamembrane domain of the receptor, but its role in Tie2 signal transduction must be still elucidated. To determine the potential binding sites for the SH2 domain of ShcA on Tie2 receptor, we created three Tie2 mutants by mutating the carboxyl-terminal Tyr residues into a Phe residue. These mutants were designed Tie2-Y1101F, Tie2-Y1107F, and Tie2-Y1112F, respectively. Tie2-3F mutant was characterized by mutations in Tyr-1101, Tyr-1107, and Tyr-1112, whereas Tyr-815 was not substituted. This mutant may give indirect indications on the role this Tyr residue. Finally, we created a kinase-dead form of Tie2 (Tie2-K584R) by mutating the Lys-854 in ATP binding site of the kinase domain into an Arg residue (Fig. 2*B*) (38). COS7 cells expressed negligible amount of Tie2, which was not phosphorylated in Tyr residues after Ang-1 stimulation (Fig. 2*C*). Tie2 constructs were transiently expressed in COS7 cells, and the encoded proteins immunoprecipitated from Ang-1-stimulated cells with anti-Tie2 Ab and immunoblotted with anti-pTyr mAb (Fig. 2*C*). Basal Tie2 phosphorylation was comparable in wild-type and Tyr-Phe mutants, whereas the K594R mutation completely abrogated the phosphorylation of the receptor.

For mapping the ShcA-binding sites, association experiments based on binding of Tie2 receptor mutants with the GST-ShcA-SH2 domain were done (Fig. 2*D*). The SH2 domain of ShcA was able to bind wild-type Tie2 receptor as well as the single mutants Tie2-Y1107F and Tie2-Y1112F. Binding was lost upon mutation of single Tyr-1101 and for the Tie2-3F (Fig.

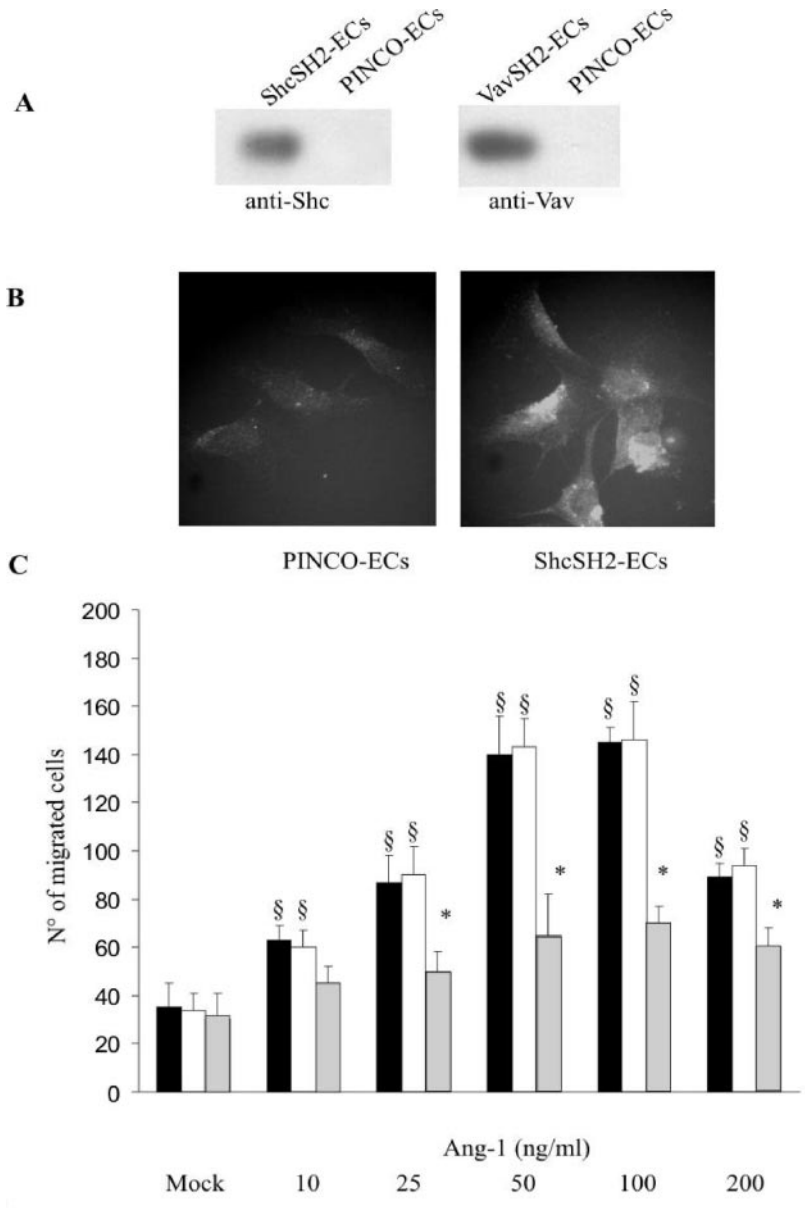

FIG. 3. **Effect of dominant-negative ShcA on Ang-1-stimulated chemotaxis of ECs.** *A*, ECs were infected with the vector PINCO alone (*left panel*) or with vector carrying the SH2 domains of ShcA or Vav, both tagged with a c-Myc epitope. Total cell lysates were separated by SDS-PAGE and immunoblotted with an anti-Shc or anti-Vav to detect. These blots are representative of four independent experiments. *B*, ECs carrying PINCO alone or ShcSH2 were seeded on collagen-coated coverslips and, after 24 h, were fixed, permeabilized, and stained with a monoclonal anti-c-Myc antibody followed by a fluorescein isothiocyanate-labeled goat anti-mouse IgG (magnification: ×400). These pictures are representative of eight stainings performed on five different batches of infected cells. The secondary Ab alone gave constant negative results (data not shown). *C*, cells were seeded in the upper wells of a 48-well microchemotaxis Boyden chamber and incubated for 7 h at 37 °C in medium containing 1% FCS. The lower wells contained the indicated concentrations of Ang-1 or mock supernatant. PINCO-ECs (*black bar*), VavSH2-ECs (*white bar*), and SchSH2-ECs (*gray bar*) migrating through a polycarbonate membrane were quantified by staining the cells with Giemsa solution, and counting was performed on a light microscope of five high power fields (×100). The results are expressed as the mean ± S.D. of three independent experiments performed in triplicate. Data were analyzed by one-way analysis of variance ($p < 0.0002$) and Student-Newman-Keuls test (*, $p < 0.05$ *versus* cells carrying vector alone or SH2Vav; §, $p < 0.05$ *versus* mock-stimulated cells).

2*D*). Tie2-K584R did not recruit the SH2 Sch domain (Fig. 2*D*). These results indicate that pTyr-1101 mediates the binding of ShcA to activated Tie2 receptor.

Inhibition of Ang-1-stimulated Migration and Sprouting by Expression of the ShcA SH2 Domain in Endothelial Cells—To examine the role of ShcA in Ang-1 signaling, ECs were infected with the retroviral vector PINCO carrying the SH2 domain of ShcA under the control of long terminal repeat promoter and the GFP under the control of cytomegalovirus promoter (ShcSH2-ECs). The ShcA SH2 domain acts as a dominant-negative mutant by competing with endogenous ShcA binding to upstream tyrosine-phosphorylated proteins (23, 32, 39, 40). As controls, ECs were infected with the vector alone carrying GFP (PINCO-ECs) or VavSH2 (VavSH2-ECs). Infection efficiency was evaluated through GFP analysis with fluorescence microscope and fluorescence-activated cell sorting, giving 85–90% of positive ECs (data not shown). Fig. 3*A* shows the immunoblot with Ab anti-SchA and anti-Vav demonstrating the transgene expression. The expression of ShcSH2 was further confirmed by immunofluorescence with a mAb against c-Myc. Fig. 3*B* clearly shows that the level of antigen detected by the anti-c-Myc Ab was higher in ShcSH2-ECs than in PINCO-ECs. Infection conditions *per se* were without effects on EC morphology and on cell cycle analyzed by propidium iodide fluorescence by FACScan flow cytometer (data not shown).

Using chemotaxis Boyden chamber, we assessed the migratory response of PINCO-ECs, ShcSH2-ECs, and VavSH2-ECs to increasing amounts of Ang-1. Ang-1 led to a significant dose-dependent increase in directed migration of PINCO-ECs (Fig. 3*B*) and VavSH2-ECs with a maximal effect at 50 ng/ml, in agreement with data reported previously (4). To distinguish the chemokinetic component of EC motility, the chemotactic gradient was abrogated by adding increasing amount of Ang-1 in the upper chamber of Boyden apparatus. The checkerboard analysis of the results obtained demonstrated the capacity of Ang-1 to increase random motility of the PINCO-ECs as well as VavSH2-ECs (Fig. 4, *A* and *B*). The expression of ShcSH2 in ECs drastically reduced their motility activity to Ang-1 (Fig. 3*B*). The checkerboard analysis indicated that the abrogation of endogenous ShcA activity mostly inhibited the chemokinetic activity of Ang-1 (Fig. 4*C*).

Spheroid aggregates of ECs sprout and invade the surrounding collagen gel forming a ring of capillary-like structures in the presence of an angiogenic inducer in a process closely

A

Upper Chamber

Lower Chamber: Ang-1 (ng/ml)	0	10	25	50
0	31±4	33±3	30±3	28±5
10	53±5	50±5	46±3	40±6
25	80±5	75±7	80±4	76±5
50	140±3	118±5	110±11	120±4

N° of migrated cells

B

Upper Chamber

Lower Chamber: Ang-1 (ng/ml)	0	10	25	50
0	27±6	31±5	34±8	30±5
10	49±7	56±5	46±3	44±7
25	78±7	77±10	84±4	72±9
50	123±7	115±8	113 ±15	129±7

N° of migrated cells

C

Upper Chamber

Lower Chamber: Ang-1 (ng/ml)	0	10	25	50
0	31±4	29±8	30±8	25±8
10	43±7	36±6	39±3	30±11
25	50±7	47±3	42±5	42±5
50	71±10	73 ±5	69 ±6	46±7

N° of migrated cells

FIG. 4. **Checkerboard analysis of Ang-1-dependent motility of PINCO-ECs (*A*), SchSH2-ECs (*B*), and VavSH2-ECs (*C*).** Ang-1-induced EC chemotaxis was evaluated by adding Ang-1 at specified concentrations in the lower compartment of Boyden chamber in the absence or presence of specified concentrations of the Ang-1 added in the upper compartment of the apparatus. Mean ± S.D. of three independent experiments was performed in triplicate.

resembling sprout formation *in vivo* (6, 41). Ang-1 was able to induce the formation of a few and large capillary-like sprouts at a concentration of 200 ng/ml, similar to those induced by stimulating pulmonary artery (42) or microvascular ECs (6). The expression of SchSH2 dominant negative molecule completely abrogated the ability of Ang-1 to promote capillary-like sprouting (Fig. 5). These results suggest that the interaction between Tie2 receptor and the adapter protein ShcA may play a role in the regulation of migration and three-dimensional organization of ECs in response to Ang-1.

Expression of ShcSH2 Does Not Affect the Protective Action of Ang-1 on EC Death—Ang-1 delays cell death associated with growth factor withdrawal or induced by irradiation or osmolarity changes (5, 43, 44). Consistent with the protective action of Ang-1 on ECs apoptosis are recent observations demonstrating that activation of the Tie2 receptor stimulates the recruitment of p85 regulatory subunit of PI 3-kinase and downstream action of the Akt signaling pathway (10, 12). To verify the involvement of ShcA in the survival vascular endothelium, PINCO-ECs, ShcSH2-Ecs, and VavSH2-ECs were serum-starved for 6 h and then maintained in fresh medium containing 0.5% FCS and 200 ng/ml Ang-1 for 12, 24, and 48 h. At the end of incubation, cellular viability was determined by MTT assay (37), which has been shown to produce results comparable with the clonogenic assay (45). Ang-1 was able to induce a 20–30% increment of cell survival after 24 h of incubation with PINCO-ECs (Fig. 6*A*) or VavSH2-ECs (Fig. 6*C*). In ECs expressing ShcSH2-ECs, the increment was similar than control cells. Surprisingly, in this cell type, the protective effect of Ang-1 was appearing earlier after 12 h of incubation (Fig. 6*B*). These results indicate that ShcA is not directly involved in the survival effect elicited by Ang-1 and that the expression of a dominant-negative mutant of the adapter protein is able to favor and anticipate the survival response in ECs.

Expression of ShcSH2 Partially Inhibits the Ang-1-dependent Tyrosine Phosphorylation of the PI 3-Kinase Regulatory Subunit p85—Because PI 3-kinase has been demonstrated to be involved in Tie2-dependent signaling pathway (11, 46) and play a role in both cell migration and survival (47), we studied the effect of the expression of ShcSH2 on Tyr phosphorylation of p85 regulatory subunit upon Ang-1 stimulation of ECs. Fig. 7 shows that the expression of this mutant partially abrogated p85 phosphorylation immunoprecipitated from ECs challenged with Ang-1. Densitometric analysis indicates that ShcSH2 in-

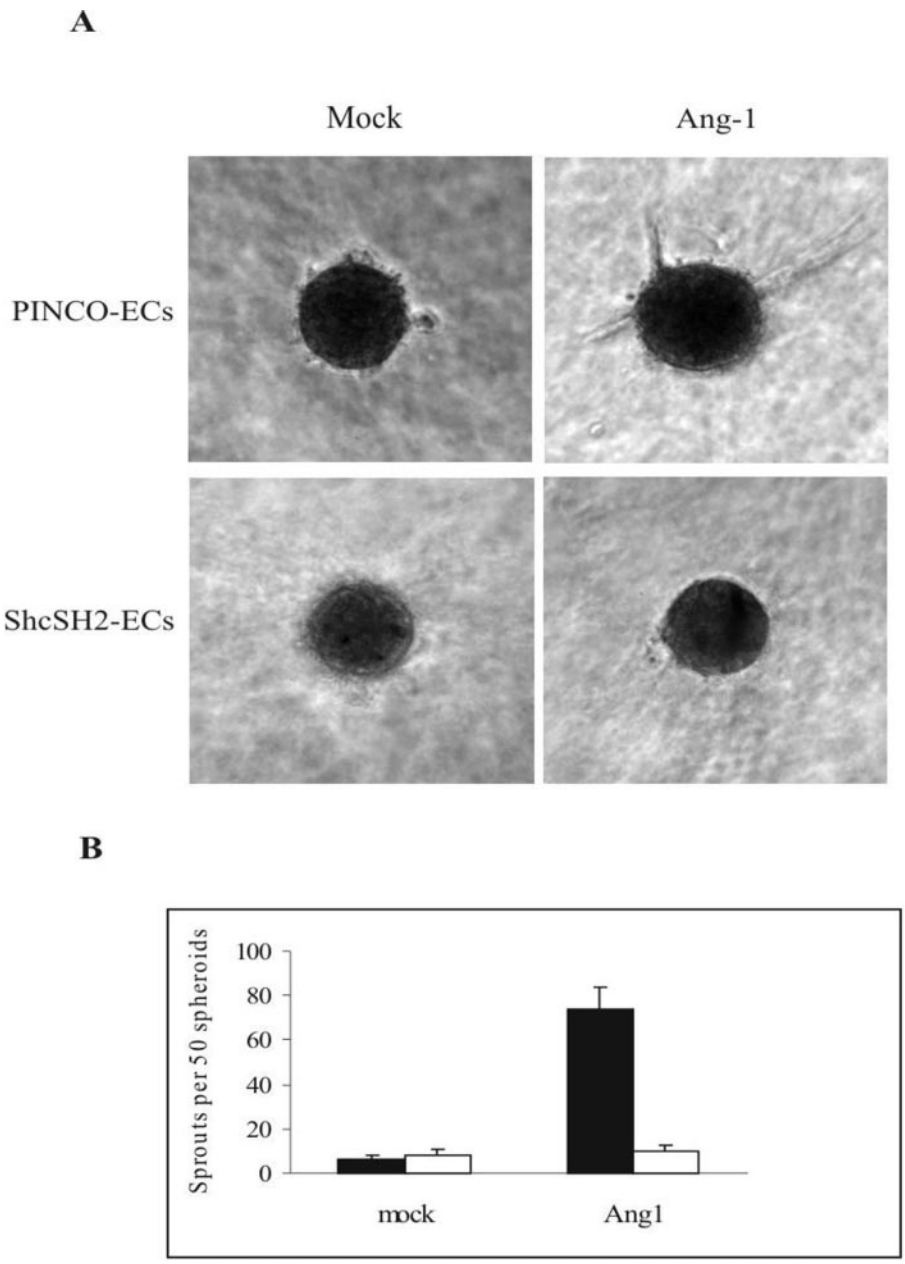

FIG. 5. **Effect of dominant-negative ShcA on Ang-1-stimulated sprouting of ECs.** *A*, EC spheroids prepared as detailed under "Experimental Procedures" were embedded in collagen gel and stimulated with mock supernatant or Ang-1 (200 ng/ml). After 24 h, photographs were taken with digital CCD Hamamatsu ORCA camera linked to an inverted microscope (magnification: ×200). *B*, the total number of capillary sprouts per 50 spheroids was counted. *Black* and *white bars* indicate, respectively, the sprout number done by PINCO-ECs and ShcSH2-ECs. Values are expressed as the mean ± S.D. of five independent experiments.

hibits ~50% of the degree of p85 phosphorylation resulting by Ang-1 challenge.

DISCUSSION

Activation of Tie2 receptor by Ang-1 has been implicated in the modulation of vascular maturation during angiogenesis (2). In this regard, Ang-1 behaves as both a survival (11, 12, 43, 44) and a motility factor (4, 11, 33, 42) and promotes EC sprout formation (6, 42) and stabilization of tubule structures in collagen matrices (5). Following ligand binding, Tie2 receptor phosphorylates at three carboxyl-terminal tyrosine residues, which act as docking sites for a number of SH2-containing effectors (3, 11). These effectors lead to the activation of distinct signaling pathways whose contribution to the biological effect elicited by Ang-1 remains largely to be determined.

The association between ShcA and endothelium-specific receptor tyrosine kinases has been described in transfected overexpressing cells where both vascular endothelial growth factor receptors 2 and 3 co-immunoprecipitated with all three ShcA isoforms (48, 49). In our study, we extend this observation to another vascular RTK and bring evidence that the adapter protein ShcA is a new binding partner of endogenous Tie2 receptor on vascular ECs. After Ang-1 stimulation, ShcA associates with Tie2 and becomes tyrosine-phosphorylated as shown by co-immunoprecipitation experiments. The association seems to be restricted to p52 isoform of ShcA, because barely any binding of the p46 and p66 isoforms could be detected, although the cells expressed high amounts of all ShcA isoforms. The different roles of ShcA isoforms in the intracellular networks are largely unknown. Indeed, evidence of a functional divergences between them had been reported previously (50, 51). For example, the presence of the amino-terminal extension in p52Shc affects the affinity of the PTB for phosphotyrosine-containing proteins, which is approximately 10 times lower for p46Shc (50), and the region comprised between residues 29 and 45 of p52Shc, which is absent in p46Shc, is responsible for the interaction of p52 (and p66Shc) with c-Src (51). Similar to our results, 52-kDa Shc isoform is the predominant substrate for the insulin receptor tyrosine kinase and the

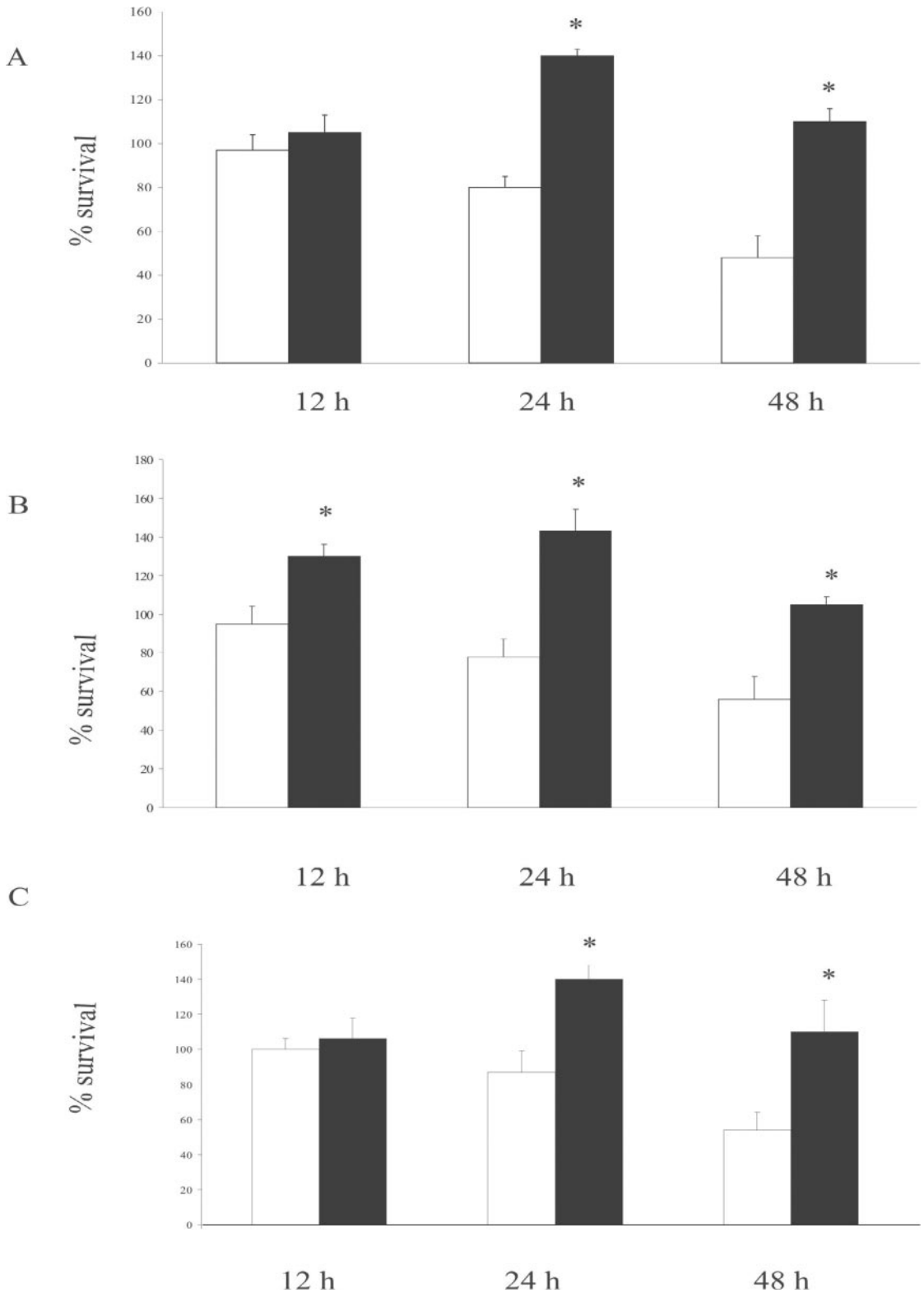

FIG. 6. **Effect of Ang-1 on survival of PINCO-ECs (*A*), ShcSH2-ECs (*B*), and VavSH2-ECs (*C*).** Cells were plated on collagen-coated 24-well plates and allowed to adhere overnight. They were starved with 0.5% FCS for 6 h, washed, and then stimulated with medium containing 0.5% FCS with (*black bar*) or without (*white bar*) Ang-1 (200 ng/ml). After 12, 24, and 48 h, washed cells were incubated with 0.2 mg/ml MTT for 2 h. The formazan product derived from the reduction of the yellow MTT tetrazolium salt by mitochondrial reductases was dissolved in dimethyl sulfoxide for 5 min. Formazan absorbance was assessed at 595 nm. Percentage of viability was calculated over the values at time zero. Mean ± S.D. of six independent experiments is shown.

polyoma middle T antigen, whereas the epidermal growth factor receptor displays similar substrate specificity for both the 52- and 46-kDa Shc species (52, 53). These differences could reflect isoform-specific downstream effectors (51) or subcellular targets (54), which are specific for different RTK. However, our results cannot exclude a role for p46 and p66 isoforms in Tie2-dependent activation of ECs being possibly phosphorylated and activated by threonine/serine kinases known to fur-

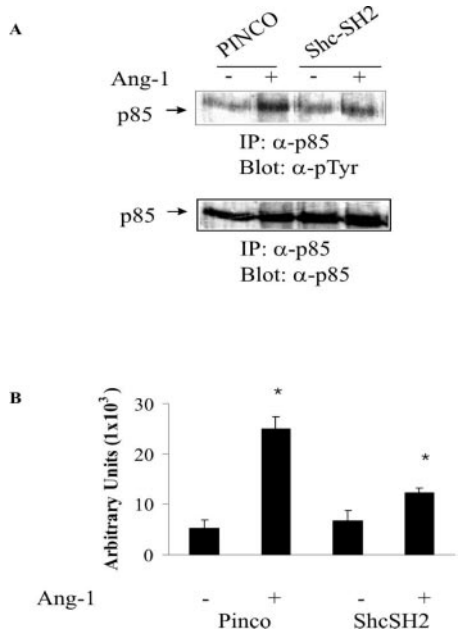

FIG. 7. **Effect of dominant-negative ShcA on Ang-1-stimulated p85 phosphorylation in Tyr residues.** *A*, ECs were infected with the vector PINCO alone or carrying SchSH2 mutants, starved, and stimulated for 10 min at 37 °C with Ang-1 (200 ng/ml). Cell lysate was immunoprecipitated by anti-p85 Ab and immunoblotted with an anti-pTyr mAb or anti-p85 Ab. *B*, densitometric analysis of tyrosine phosphorylation of p85. Mean ± S.D. of four experiments is shown. Data were analyzed by one-way analysis of variance ($p < 0.001$) and Student-Newman-Keuls test. *, $p < 0.05$ *versus* unstimulated cells.

ther modulate the functions of these adaptors (55).

The data presented here suggest that at least SH2 domain is necessary for the recruitment of ShcA to Ang-1-activated Tie2. In particular, SH2 domain associates pTyr-1101. ShcA SH2 domain recognizes pTyr in the context of COOH-terminal residues pTyr-hydrophobic-*XXX*-hydrophobic (56). Tyr-1101 of Tie2 corresponds to this optimal binding site because it is followed by the sequence VNTTL with a hydrophobic residue at +1 and +5 position. Preliminary pull-down experiments suggest that also the PTB domain may be recruited to the receptor through pTyr-1101 and pTyr-1112. However, neither of them shows the canonical motif for Shc PTB domain (NP*X*PY (57)), suggesting that this *in vitro* observation requires further investigations.

To examine the role of ShcA in Ang-1 signaling, ECs were infected with a retroviral vector carrying the SH2 domain of ShcA. The ShcA SH2 domain acts as a dominant-negative mutant by inhibiting phosphorylation of ShcA and specifically suppressing the ShcA pathway (23, 32, 39, 40). Expression of ShcSH2 leads to a reduction of Tyr phosphorylation of p85 subunit of PI 3-kinase, an inhibition of chemotaxis, and sprouting induced by Ang-1 in ECs but was ineffective in inhibiting the protective action of Ang-1 on EC death. These data indicate that the SH2-mediated recruitment of ShcA to Ang-1-activated Tie2 is required to selectively regulate motility and morphogenesis of ECs but not their survival.

There is substantial evidence that ShcA regulates cell motility (58–62) through the activation of MAPK that is known to directly phosphorylate and activate myosin light chain kinase, leading to phosphorylation of myosin light chains and promoting the cytoskeleton contraction necessary for cell movement (63, 64). In this perspective, SchA is mainly involved in integrin-mediated and growth factor-mediated random cell motility (61), which is in agreement with the checkerboard analysis of EC motility stimulated by Ang-1. Actually, Ang-1 activates Ras and MAPK (65)[2] and induces both chemotaxis and chemokinesis of ECs. The overexpression of ShcA-SH2 domain mainly inhibited random rather than directionally persistent EC motility.

Ang-1 induces the formation of capillary-like sprouts in an *in vitro* sprouting assay that is independent of cell proliferation (66). In this morphogenetic process, ECs are embedded in a gel of extracellular matrix and therefore integrin-mediated outside-in signals are crucial in vessel formation and remodeling (67). Because ShcA is recruited by the cytosolic tails of integrins (60, 68, 69) as well as by Ang-1-activated Tie2, it is stimulating to hypothesize that this adaptor integrates signals coming from these receptors leading to capillary sprouting. In this respect, a strict cooperation between $\alpha_v\beta_3$ integrin and tyrosine kinase vascular endothelial growth factor receptor-2 has been proposed as an important mean to regulate migratory response of ECs at the tip of sprouting vessels (70, 71).

PI 3-kinase is generally involved in cell migration and survival (47) and has been demonstrated to be necessary for Ang-1-stimulated motility (11, 46) as well as for its cytoprotective effect (5, 10–12). The overexpression of SchA-SH2 mutant partially reduces the Tyr phosphorylation of the regulatory p85 subunit of PI 3-kinase induced by Ang-1. p85 subunit binds the Tyr-1101 of Tie2 (10–12), the same docking site that interacts with the SH2 domain of ShcA. Furthermore, SchA may trigger a survival program (26, 72) through PI 3-kinase activation (26, 73, 74). Therefore, the overexpression of SchA-SH2 may compete for the binding of p85 to Tie2 or inhibit the direct activation of PI 3-kinase by phosphorylated Sch. Because PI 3-kinase is involved in regulation of both migration and survival, one could expect that the inhibition of p85 phosphorylation in ECs carrying SchA-SH2 is associated with an inhibition of both activities induced by Ang-1. On the contrary, our results clearly establish that SchA-SH2 exclusively affects EC motility stimulated by Ang-1. This discrepancy may be explained by the requirement of different thresholds of activation, being a middle p85 phosphorylation sufficient to address the p110 catalytic subunits toward a cytoprotective program. This is reminiscent of the observation that different biological responses elicited by hepatocyte growth factor correlate with different threshold levels of PI 3-kinase and Sch (75). Alternatively, we may speculate that the role of p85 in this context is independent from the catalytic activity of PI 3-kinase (76).

Our data cannot completely exclude that SchA may participate in the survival signals of Ang-1. Actually, we cannot rule out that Sh2-SchA dominant negative allows the PTB-mediated interaction between Tie2 and SchA. Alternatively, the independence of cytoprotective action of Ang-1 from ShcA may reflect different responsiveness of Tie2. For instance, vascular endothelial growth factor receptor 2-mediated activation of PI 3-kinase results in migration or survival. These activities are reciprocally exclusive and depend on the association of the receptor with $\alpha_v\beta_3$ integrin or VE-cadherin with $\alpha_v\beta_3$ integrin being permissive for endothelial motility and VE-cadherin being permissive for survival (70, 71, 77, 78). Therefore, a possibility is that Tie2 forms complexes with other transmembrane proteins, which contribute in the different assembly of cytoplasmic transduceosome (79) including the modulation of SchA-mediated effects.

Data obtained from knock-out mice reveal that ShcA plays an important role in vascular development (27). Null mice have deregulated adhesive contact of the endothelium with adjacent extracellular matrix components and mural cells (pericytes and smooth muscle cells). This phenotype partially mimics that of

[2] E. Audero and F. Bussolino, unpublished results.

Ang-$1^{-/-}$ or Tie$2^{-/-}$ mice (28, 29) in which maturation and remodeling of the primitive vascular network is impaired.

Therefore, the binding of the adapter molecule ShcA to the Tie2 receptor may have an important role *in vivo* in the regulation of the signaling that leads to the migration and organization of ECs elicited by Ang-1.

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Mechanisms of Signal Transduction:

Adaptor ShcA Protein Binds Tyrosine Kinase Tie2 Receptor and Regulates Migration and Sprouting but Not Survival of Endothelial Cells

Enrica Audero, Ilaria Cascone, Fabrizio Maniero, Lucia Napione, Marco Arese, Luisa Lanfrancone and Federico Bussolino

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