The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo

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The ShcA gene products have served as a model for the analysis of phosphotyrosine-recognition domains, and for the functions of docking proteins during tyrosine kinase signaling. Here we show that ShcA is primarily expressed in the cardiovascular system during early mouse embryogenesis and regulates both heart development and establishment of mature blood vessels. Targeted mutation suggests that the ShcA adaptor is a pivotal target of tyrosine kinases that selectively potentiates activation of the MAP kinase pathway in the remodeling vasculature. Biochemical analysis of mutant cells shows that ShcA sensitizes cells to growth factor-induced MAP kinase activation, and also organizes cytoskeletal rearrangement in response to the extracellular matrix. ShcA may therefore orchestrate complex interactions within the vascular compartment by rendering cells permissive to respond to soluble and adhesive external cues.

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The ShcA proteins are phosphorylated on tyrosine in response to a range of extracellular signals, and have been widely studied for their ability to form multiple phosphotyrosine [pTyr]-dependent complexes, and thus to serve a docking function in tyrosine kinase signaling (for review, see Bonfini et al. 1996; Pawson and Scott 1997). Mammalian ShcA encodes three overlapping polypeptides of 46, 52, and 66 kD, that are produced through alternative splicing and differential use of translation initiation sites, and differ only in the length of their amino-terminal extensions (Pelicci et al. 1992; Migliaccio et al. 1997). These ShcA proteins possess two distinct pTyr recognition modules. At their carboxyl terminus they have an SH2 domain (Songyang et al. 1994), and toward the amino terminus they contain a pTyr-binding (PTB) domain, which recognizes phosphorylated motifs with the consensus sequence dXNXY [in which d is a large aliphatic residue] (van der Geer et al. 1996). This motif is found in a number of activated receptor tyrosine kinases (RTKs), and in cytoplasmic proteins such as SH2-containing 5’-inositol phosphatase (Damen et al. 1996). The ShcA PTB domain can also bind acidic phospholipids, which may assist in the recruitment of ShcA proteins to membranes (Zhou et al. 1995; Ravichandran et al. 1997). ShcA proteins physically associate with a variety of activated RTKs, primarily through their PTB domain, and consequently become phosphorylated at Tyr residues 239, 240, and 317 (on the basis of human 52-kD ShcA isoform) (Salcini et al. 1994; Gotoh et al. 1996; van der Geer et al. 1996). Both Tyr-239 and Tyr-317 are followed at the +2 position by an Asn residue, forming an optimal binding site for the SH2 domain of the adaptor Grb2 (Rozakis-Adcock et al. 1992; Songyang et al. 1994; van der Geer et al. 1996). The Ras guanine nucleotide exchange factors Sos1 and Sos2 are recruited into these complexes through their association with the SH3 domains of Grb2, providing a mechanism through which ShcA phosphorylation can lead to activation of the Ras–MAP kinase [MAPK] pathway (Rozakis-Adcock et al. 1992). ShcA can also associate, either directly or via Grb2, with the docking protein Gab-1, that in turn binds PI 3’ kinase (Ingham et al. 1998). Additional ShcA partners have been described, including SH3-containing proteins and adaptins (Weng et al. 1994; Matoskova et al. 1995; Okabayashi et al. 1996). 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. ShcA has been associated with JNK activation in B cells (Hashimoto et al. 1999), and the p66 ShcA isoform appears to have a unique role in regulating stress responses (Migliaccio et al. 1999).
ShcA is widely expressed in cultured cells and adult mouse tissues, and is phosphorylated in response to a large number of physiological stimuli (for review, see Bonfini et al. 1996). Two closely related mammalian genes, ShcB/Sck and ShcC/N-Shc, are primarily expressed in the nervous system (O’Bryan et al. 1996; Pelicci et al. 1996; Nakamura et al. 1998), and a single Shc gene (dshc) has been found in Drosophila (Lai et al. 1995), although the invertebrate gene product is significantly less complex than mammalian ShcA isoforms. To explore the biological and biochemical functions of ShcA proteins, we have introduced a mutation into the mouse ShcA gene. Unexpectedly, we find that ShcA is primarily expressed in the developing cardiovascular system and mediates pTyr signaling in mammalian heart development and angiogenic remodeling. Analysis of mutant cells indicates that ShcA has multiple functions, including sensitizing cells to small increases of growth factors and regulating cytoskeletal reorganization by adhesion receptors. Using an antibody that specifically recognizes the phosphorylated-activated Erk MAPK we detected high levels of MAPK activity in tissues such as the cardiovascular system and limb buds in wild-type embryos. Strikingly, MAPK activation is specifically impaired in regions of ShcA-deficient embryos that undergo angiogenic remodeling. ShcA therefore appears pivotal in the multiple signaling events required to establish a mature vasculature.

Results

Targeted disruption of the mouse ShcA gene

A targeting vector was designed to replace 2 kb of the ShcA gene with a PGK-neomycin resistance cassette (neoR) by homologous recombination in R1 mouse ES cells (Fig. 1A). The deleted genomic sequence contains the first two coding exons (exons 2 and 3), including the translational initiation sites for all three ShcA proteins and sequences encoding the amino-terminal half of the PTB domain (residues 1–80 of p52). Thus, any polypeptide expressed from the targeted (ShcAex2/3) locus would lack a functional PTB domain, and should therefore be defective for signaling activity. Southern blot
Mice heterozygous for the ShcA<sup>Δex2/3</sup> allele have not shown any detectable phenotype. However, screening of >800 offspring from heterozygous intercrosses failed to identify any viable homozygotes. To determine the point at which ShcA mutant embryos died, embryos from heterozygous intercrosses were examined. ShcA<sup>Δex2/3</sup> allele-specific PCR analysis (Fig. 1C) of embryos collected from embryonic day E9.5 to E11.5 showed that homozygotes were obtained with approximately the expected Mendelian frequency (Table 1). ShcA<sup>Δex2/3</sup> mutant embryos had a normal morphology at E9.5 (Fig. 2). By E10.5, mutant embryos had an enlarged pericardium and abnormal cardiac contractions, but were equivalent in size to wild-type embryos. By E11.5, all ShcA<sup>Δex2/3</sup> homozygotes appeared necrotic with pale yolk sacs, and their heart and cardiac outflow tracts were congested with blood. No homozygous mutant embryos were identified beyond E12. Examination of other areas of the embryos indicated that development of the placenta and limb buds of homozygous mutant embryos was grossly normal when compared with their littermate controls. Thus, ShcA<sup>Δex2/3</sup> mutants die by E11.5 with evidence of a gross cardiovascular defect.

To analyze the effects of the Δex2/3 mutation on ShcA expression, we performed Northern and Western blot analysis. The normal 2.8 and 3.4 kb ShcA RNA transcripts and three ShcA polypeptides (46, 52, and 66 kD) could be detected as early as E9 (Fig. 1D,E), but were absent in homozygous mutants (Fig. 1D,E). However, immunoblotting with antibodies to the carboxy-terminal region of ShcA identified a small amount of a novel 40-kD protein in +/+ and −/− embryos. The amount of this protein in mutant embryos was ~2% of ShcA protein in wild-type embryos. RT–PCR analysis suggested that the mutant locus yields a transcript that initiates at the start site from the endogenous 2.8-kb mRNA, and is aberrantly spliced into the neo<sup>R</sup> cassette and subsequently into the fourth exon of the ShcA gene (data not shown). Coupled in vitro transcription/translation identified Met-134 as the likely initiation codon for the p40 protein. Thus, the ShcA<sup>Δex2/3</sup> mutant allele encodes a low level of a 40-kD protein that lacks residues critical for PTB domain function.

**Table 1.** ShcA homozygote mutants die before E11.5 of gestation

| Developmental stage | No. of embryos |
|---------------------|----------------|
|                     | total (%) | +/− (%) | +/− (%) | −/− (%) |
| E8.5                | 21 (100)  | 4 (19)  | 12 (57) | 5 (24)  |
| E9.5                | 121 (100) | 33 (27) | 60 (50) | 28 (23) |
| E10.5               | 324 (100)| 82 (25) | 171 (53)| 71 (22) |
| E11.5               | 84 (100)  | 27 (29) | 50 (53) | 17 (18) |
| E13.5<sup>b</sup>   | 60 (100)  | 15 (25) | 31 (52) | 0       |

<sup>a</sup>All embryos were necrotic.
<sup>b</sup>Fourteen embryos had degenerated and were being resorbed.

**ShcAΔex2/3 mutants show abnormal heart development**

Whole mount examination of E10 mutant embryos showed that the heart had advanced to the point where normal right-sided cardiac looping was completed. Expression of cardiac markers such as cardiac actin and myosin light chains 2A and 2V was normal (data not shown), but the left ventricle was thin walled and irregular in shape, a defect that became more pronounced later in development.

To investigate this phenotype in more detail, serial transverse and sagittal sections of embryos from E8.5 to E11 were analyzed. Beginning at E9.5, a difference was observed in the normal pattern of finger-like projections formed by cardiomycocytes, known as trabeculae, and by E10.5, the trabeculae were severely reduced in the ShcA<sup>Δex2/3</sup> mutant embryos (Fig. 2C), as compared with wild-type littermates (Fig. 2B). The thin myocardium associated with reduced trabeculation in ShcA<sup>Δex2/3</sup> mutants likely contributes to poor heart contractility, leading to congestion of blood in the heart chamber. In addition, the endocardial cushions were poorly developed in mutant embryos (Fig. 2B,C), although the intraventricular septum appeared normal (Fig. 2D,E). Owing to the broad expression of ShcA in adult tissues and its potential involvement in many tyrosine kinase systems, this specific cardiac phenotype was unexpected. We therefore analyzed the pattern of ShcA expression during early embryonic development.

**ShcA is predominantly expressed in the developing cardiovascular system**

Whole-mount RNA in situ hybridization analysis of E9 wild-type embryos indicated that ShcA is primarily expressed in the developing cardiovascular system (Fig. 3). ShcA transcripts were detected at high levels in the heart (Fig. 3A), and in the region of the paired dorsal aorta. This latter staining was continuous with the branching arteries that traverse bilaterally through the branchial arches toward the aortic sac (Fig. 3B), and with the intersomitic vessels (Fig. 3C). This pattern of ShcA transcripts persists at E9.75. However, expression by this stage was more widespread, and especially pronounced in the craniofacial mesenchyme (Fig. 3D).

To obtain a more detailed analysis of ShcA expression, a histological examination of serial sections through these embryos was undertaken. ShcA RNA was identified in the head mesenchyme, in the region in which the anterior carotid arteries develop (Fig. 3E), in the endothel-
ShcA regulates cardiovascular development

ShcAΔex2/3 mutants have defects in angiogenesis and cell–cell contacts

The abundant cardiovascular expression of ShcA is consistent with the failure in heart development in mutant embryos, and raised the possibility that ShcA might have a broader role in vessel morphogenesis. We therefore analyzed wild-type and ShcAΔex2/3 mutant mice carrying a Tek–lacZ transgene, in which β-galactosidase (β-Gal) provides a specific marker for endothelial cells [ECs] (Dumont et al. 1994). Whole-mount staining with X-Gal revealed no absolute block to the early assembly of blood vessels derived by vasculogenesis or angiogenesis in ShcAΔex2/3 mutants [Fig. 4A,B]. However, the β-Gal staining pattern was significantly less complex in the mutant embryos, particularly in the head. In addition, the ECs forming the endocardium in the mutant heart appeared thinner and more elongated [Fig. 4D]. Defective vasculature was also apparent in cross sections of the yolk sac, which showed large and uniformly sized lumens [Fig. 4F]. Discrete structures indicative of angioblast-derived blood islands appeared highly disorganized [Fig. 4F].

We used antibodies to PECAM-1 to compare vascular development throughout the embryo. ShcAΔex2/3 mutants have a similar overall vascular patterning to age-matched wild-type embryos, but showed dilatation and decreased complexity of the primary head vessels at E10.5 [Fig. 4G,H]. For example, the anterior carotid arteries in the ShcA mutant heads were expanded in size and had a more simplified morphology than in wild-type embryos.

The decreased complexity of the vascular architecture and increased vessel size observed in ShcAΔex2/3 mutants indicates a perturbation in later step[s] of vascular development involving angiogenic remodeling (for review, see Risau 1997). During vessel enlargement and angiogenic sprouting, signals from ECs promote proliferation of VSMC precursors and their comigration along angiogenic sprouts; VSMCs in turn regulate EC behavior. Thus, the mature vascular network is stabilized by surrounding VSMCs, pericytes [PCs], and fibroblasts, in a fashion regulated by the extracellular matrix [ECM] and growth factors [for review, see Folkman and D’Amore 1996; Hirschi and D’Amore 1997]. To test whether the defect in vessel maturation in ShcAΔex2/3 mutants was accompanied by aberrant VSMC organization, these cells were examined by immunostaining with antibodies to smooth muscle α-actin [SMA]. In mutant embryos, SMA-positive signals were consistently reduced throughout the entire vascular system compared with
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**Figure 3.** Expression pattern of ShcA RNA in wild-type embryos. (A) E9.0 whole-mount embryo. ShcA transcripts are abundant within and around the paired dorsal aortae, the contiguous omphalomesenteric artery, and the vitelline artery, as well as in the branching intersomitic arteries. Staining is clear in the ventricular chamber of the heart, and in the first, second, and third branchial arch arteries. Abundant transcripts are also evident around head vessels. (B) Higher magnification of A, branchial arch region. Note the expression of ShcA transcripts in and around aortic arteries that traverse through the branchial arches. (C) Higher magnification of the branching intersomitic vessels (arrows) as in A. (D) E9.75 whole-mount embryo. Transcripts are now detectable in the cranial mesenchyme. Scattered staining is obvious in the head. Expression in the dorsal aorta, heart, and otic vesicle remains detectable. (E–I) Rostral to caudal transverse sections of the E9.0 embryo shown in D. High levels of ShcA transcripts are found in the head mesenchyme in which the anterior carotid arteries develop [E], in and around the dorsal aorta [F, H, I] and branchial arch arteries [F]. Transcripts are barely detectable in the anterior cardinal vein [F] and common cardinal vein [H]. Prominent expression is also observed in the endocardium and surrounding mesenchymal cells within the heart [G], in the vitelline artery but not in the umbilical vein [I], in the endothelium of intersomitic vessels [arrowheads] and neighboring cells [F]. Patchy staining within the neural tube is indicated by arrows [J, I, II, III] First, second, and third branchial arch arteries; [A] aorta; [RA] branchial arch, [CV] cardinal vein; [DA] dorsal aorta; [EC] endocardium; [Fa] facial cranial mesenchyme; [FL] forelimb; [H] heart; [HV] head vein; [M] myocardium; [NT] neural tube; [OA] omphalomesenteric artery; [OV] otic vesicle; [PNCP] peri-neural capillary plexus; [R] rhombomere; [S] somites; [TV] telencephalic vesicle; [UV] umbilical vein; [V] ventricle; [VA] vitelline artery.

ShcA is phosphorylated on tyrosine and associated with signaling proteins during embryogenesis

A number of mammalian RTKs and their ligands are specifically expressed in vessel cells and are important for cardiovascular development. RTKs, including ErbB2/B3/B4 and Tie-1/Tie-2 (Tek), were expressed to normal levels in ShcAΔex2/3 mutants [data not shown]. To test whether ShcA may be a direct target for activated RTKs involved in formation of the vascular system, lysates from wild-type E10 embryos were immunoprecipitated with antibodies to ShcA, a distinct adaptor protein Nck, pTyr, or control serum, and blotted with anti-pTyr antibodies [Fig. 5A]. The ShcA isoforms were all strongly phosphorylated on tyrosine, and coprecipitated with a series of larger pTyr-containing proteins (~120, 140, and 180 kDa), which potentially correspond to activated RTKs. These results indicate that ShcA proteins are ac-
ShcA mutant cells and embryos show impaired sensitivity to growth factors

Phosphorylated ShcA proteins from E10 lysates coprecipitated with Grb2 (data not shown), raising the possibility that ShcA might influence signaling to the Ras/MAPK pathway in vivo. We therefore investigated the ability of PDGF-BB and EGF to activate MAPK in mouse embryo fibroblasts (MEFs) derived from ShcA-deficient embryos. This assay may be physiologically relevant because PDGF signaling, for example, is important for the formation of vessel walls (Hellstrom et al. 1999). MEFs were serum deprived, and stimulated with PDGF-BB (25 ng/ml) or EGF (50 ng/ml). Erk2 activation was monitored by a gel-shift assay, which identifies the active MAPK pathway in vivo. We therefore investigated the ability of PDGF-BB and EGF to activate MAPK in mouse embryo fibroblasts (MEFs) derived from ShcA-deficient embryos. This assay may be physiologically relevant because PDGF signaling, for example, is important for the formation of vessel walls (Hellstrom et al. 1999). MEFs were serum deprived, and stimulated with PDGF-BB (25 ng/ml) or EGF (50 ng/ml). Erk2 activation was monitored by a gel-shift assay, which identifies the active MAPK pathway in vivo. We therefore investigated the ability of PDGF-BB and EGF to activate MAPK in mouse embryo fibroblasts (MEFs) derived from ShcA-deficient embryos. This assay may be physiologically relevant because PDGF signaling, for example, is important for the formation of vessel walls (Hellstrom et al. 1999). MEFs were serum deprived, and stimulated with PDGF-BB (25 ng/ml) or EGF (50 ng/ml). Erk2 activation was monitored by a gel-shift assay, which identifies the active MAPK pathway in vivo. We therefore investigated the ability of PDGF-BB and EGF to activate MAPK in mouse embryo fibroblasts (MEFs) derived from ShcA-deficient embryos. This assay may be physiologically relevant because PDGF signaling, for example, is important for the formation of vessel walls (Hellstrom et al. 1999). MEFs were serum deprived, and stimulated with PDGF-BB (25 ng/ml) or EGF (50 ng/ml). Erk2 activation was monitored by a gel-shift assay, which identifies the active MAPK pathway in vivo. We therefore investigated the ability of PDGF-BB and EGF to activate MAPK in mouse embryo fibroblasts (MEFs) derived from ShcA-deficient embryos. This assay may be physiologically relevant because PDGF signaling, for example, is important for the formation of vessel walls (Hellstrom et al. 1999). MEFs were serum deprived, and stimulated with PDGF-BB (25 ng/ml) or EGF (50 ng/ml). Erk2 activation was monitored by a gel-shift assay, which identifies the active MAPK pathway in vivo.
phosphorylated species of this MAPK isoform. In both wild-type and ShcA mutant cells, Erk2 was activated to a similar extent and with similar kinetics. Maximal Erk2 activation was apparent within 5 min in both cell types, and in response to both growth factors (Fig. 5C). Anti-pTyr blotting of whole cell lysates revealed a similar overall response to PDGF and EGF in wild-type and mutant MEFs (data not shown).

However, mutant and wild-type cells had very different dose-response curves with respect to Erk2 activation (Fig. 5D). Addition of EGF at 0.5 ng/ml fully activated MAPK in wild-type cells, but only induced 20%–30% increase in Erk2 activation in mutant cells. Full Erk2 activation in mutant cells required at least 50-fold higher concentrations of EGF. This impaired sensitivity to EGF was observed in five different cultures of ShcA mutant fibroblasts derived from independent litters (data not shown). Similarly, MAPK was optimally activated by 1 ng/ml of PDGF-BB in wild-type cells, but required at least 25 ng/ml for strong activation in mutant cells. To test whether this decreased sensitivity to growth factor stimulation resulted solely from the loss of wild-type ShcA proteins, mutant cells were transfected with a mouse cDNA encoding the p52 and p46 ShcA isoforms. Mutant cell lines stably expressing the p52 and p46 proteins to ~70% the level seen in wild-type cells (Fig. 5B) recovered full Erk2 activation at low growth factor concentrations. The extent of MAPK activation was confirmed in each case by measuring in vitro kinase activity (data not shown). These data indicate that wild-type p52/46-kD ShcA proteins are required for cultured cells to respond to low concentrations of growth factors.

Whole-mount immunostaining with phosphospecific Erk MAPK antibodies demonstrates selective loss of MAPK activation in the cardiovascular system of ShcA mutant embryos.

The preceding data obtained from MEFs suggest that...
ShcA may sensitize cells to growth factor signaling in vivo. To compare MAPK activation in wild-type and mutant embryos, we used an antibody to the doubly phosphorylated form of MAPK (dp-MAPK) in a whole-mount analysis. A similar approach has been used previously in *Drosophila* to correlate the dynamic pattern of MAPK activation with RTK expression (Gabay et al. 1997). E10.5 wild-type embryos showed intense dp-MAPK staining associated with head vessels and the tips of vessel sprouts that was markedly reduced in *ShcA* mutant embryos (Fig. 6A–D). This reduction appeared selective, as both wild-type and mutant embryos had comparable dp-MAPK staining in other regions of the head such as the mid/hindbrain junction (Fig. 6A,B). To examine whether MAPK activation might be a cause or a consequence of the cardiovascular defects in *ShcA* mutants, we analyzed embryos for MAPK activation at E9.5, prior to significant manifestation of phenotypic abnormalities. Compared with wild-type embryos, *ShcA* mutants exhibited a severe and specific reduction of dp-MAPK staining in regions that correlate with cardiovascular development and the normal pattern of ShcA expression (Fig. 6E). Reduced MAPK activation was evident in the heart ventricle (Fig. 6F,G) and in the dorsal side of the embryos around the termini of intersomitic vessels (Fig. 6H,I), although the ventral regions of the trunk and intersomitic vessels were less affected. Double labeling with dp-MAPK and PECAM-1 confirmed that vessel development at this stage was grossly normal, despite the regional reduction of MAPK activation in mutant embryos (Fig. 6J,K). The restricted expression of ShcA in cells of the vascular system is therefore required for their ability to appropriately activate the MAPK pathway. ShcA deficiency may therefore desensitize the vascular precursor cells to external stimulation required for angiogenic remodeling. Taken with the specific expression of ShcA in the cardiovascular system, these results argue that the phenotype of ShcA mutant embryos reflects a direct role for ShcA in cardiovascular development.

Loss of ShcA affects fibronectin-induced MAPK activity, focal complex distribution and the actin cytoskeleton

*ShcA* mutant embryos exhibited adhesion defects in yolk sac (Fig. 4E,F), in the heart (Fig. 4O,P) and around dorsal aorta (Fig. 4K–N). Because activation of some integrins triggers ShcA phosphorylation and activation of the MAPK cascade (Wary et al. 1996), we compared the behavior of wild-type or mutant cells plated on fibronectin (FN). Both cell types adhered to FN-coated plates within 10 min. MAPK activation was observed in wild-type serum-starved cells plated on FN, but not on poly-l-lysine (Fig. 5E); *ShcA* mutant MEFs plated on FN showed a reduced level of Erk activation as measured by blotting with phosphospecific Erk antibodies (Fig. 5E), and is partially rescued by the expression of wild-type p52 ShcA [data not shown].

Compared with wild-type cells, ShcA-deficient MEFs also showed significant alterations in the organization of their focal contacts and actin stress fibres, as revealed by immunostaining with antibodies to paxillin (Fig. 7A,B) or vinculin [data not shown] and with rhodamine–phalloidin to identify F-actin (Fig. 7B). Double labeling with

**Figure 6.** MAPK activation is selectively reduced in the developing cardiovascular system of *ShcA* mutant embryos. (A–D) Pattern of Erk activation in E10.5 heads. (E) Higher magnification views of the boxed areas of A and B, respectively. Intense dp-Erk staining was specifically detected along wild-type head vessels [A, arrows in C], which was much reduced in the mutant [B,D]. Sites of activated MAPK [circles] that were prominent in the tips of wild-type vessel sprouts (C) were extremely weak in *ShcA* mutant [D]. Comparable staining was observed around the mid-hindbrain region [asterisks] in both wild-type and mutant embryos. (E–K) Comparison of Erk activation in whole E9.5 embryos labeled with anti-dp-Erk [brown] and anti-PECAM-1 [blue, J and K only] antibodies. In mutants, dp-Erk staining was reduced in the cardiovascular system but unaffected in other regions, including the developing limbs and ventral side of the trunks [E]. In mutants, dp-Erk expression was reduced in the left ventricle of the heart [wild-type, F, Mut, G] and around the dorsal side of the trunk (asterisks and arrows), although staining toward the proximal ISV was less affected [arrows] [Wt, H and J, Mut, I and K]. Double labeling for dp-Erk and PECAM revealed the normal appearance of ISV at E 9.5 in both wild-type [J] and mutant [K], but with reduced MAPK activity around distal ISVs in the mutant [arrows]. (B) Branchial arches; (H) heart; (L) limb.
phalloidin (red) and anti-paxillin antibody (green) showed that wild-type cells plated on FN contained lamellipodia, with sparsely arranged, polarized actin stress fibres terminating in coarse paxillin-positive structures (Fig. 7B). In contrast, ShcA mutant MEFs had intensely staining actin bundles around the entire cell periphery, with fine paxillin-positive focal complexes that showed an atypical radial distribution. Polarized flat protrusions resembling lamellipodia were only detected in 15%–20% of the mutant cells. ShcA mutant cells were rounded, with extensive membrane blebbing. These morphological and cytoskeletal defects were suppressed by expression of the p52/46 ShcA isoforms in the mutant cells (Fig. 7A,B). Thus, ShcA potentially plays a significant role in fibroblasts in organizing cytoskeletal responses to ECM components such as FN.

Discussion

ShcA activity is required for maturation of blood vessels and heart development

Our results indicate that during early embryogenesis ShcA is specifically expressed in cells of the developing cardiovascular system, which consequently become sensitized to signals that direct heart development and angiogenesis. Although the principal vascular progenitors are properly induced and assembled in ShcA mutants, the vessels show selective defects in their subsequent organization into a mature vascular network. This is most evident in the yolk sac and head vasculature, in which vessels are dilated and remain relatively uniform in size. ShcA mutant embryos exhibited adhesion defects in yolk sacs, in the heart, and in major blood vessels. Ultrastructural analysis of the dorsal aorta reveals a significant reduction in the contacts of ECs with periendothelial cells and the surrounding mesenchyme. Similarly, support cells around endothelial tubes are poorly organized. Thus, ShcA appears important in the reciprocal signaling between ECs and support cells required for angiogenic remodeling and heart development. Consistent with this view, ShcA proteins are highly phosphorylated on tyrosine at E10, a critical time for both angiogenesis and vessel stabilization, and are complexed with other phosphoproteins and with Grb2.

Interesting, despite early cardiovascular expression and tyrosine phosphorylation [Fig. 5a; data not shown] of the 66-kD ShcA isoform in the developing embryos, mice lacking only p66 are viable [Migliaccio et al. 1999]. It is therefore probable that the 46/52-kD ShcA proteins are the isoforms essential for embryonic development. The ShcA<sub>ex2/3</sub> mutant allele encodes a very low level of 40-kD protein that lacks a function PTB domain. Although we cannot exclude the possibility that the 40-kD mutant protein retains some biological activity, several observations argue that this truncated protein is unlikely to cause a dominant-negative effect or to have significant signaling functions. First, heterozygotes have no apparent phenotype. In addition, quantitative analysis showed that the truncated protein is only expressed at 2% of the wild-type ShcA level. Furthermore, the 40-kD polypeptide lacks a functional PTB domain, which has been shown biochemically to be important for ShcA-signaling function. Recently, a genetic screen in Drosophila identified mutations in the <em>dshc</em> locus and showed that a point mutation in the PTB domain completely abolished <em>dshc</em> function, resulting in the same phenotype as the null mutation. This indicates that an intact PTB domain is essential for <em>dshc</em> activity [Luschnig et al. 2000]. Most significantly, the defects in growth factor signaling and cytoskeletal organization in ShcA mutant cells were rescued by re-expression of the wild-type 46/52-kD pro-
teins, indicating that at the cellular level the mutation causes a strong loss of function.

**Mutations in ShcA, specific RTKs or their ligands cause similar cardiovascular defects**

The phenotypes of embryos lacking endothelial RTKs or their ligands highlight the importance of reciprocal signaling between EC and VSMC compartments. Embryos deficient for the Tie2/Tek receptor [Dumont et al. 1994; Sato et al. 1995], its ligand Ang-1 [Suri et al. 1996] and the Flt4/VEGFR3 RTK [Dumont et al. 1998] have defects in the later stages of vascular patterning that are very similar to those observed in ShcA mutants, and are linked to a failure to recruit VSMC and PC precursors to developing vessels. The PDGF family is also important for vascular wall assembly [Soriano 1994; Schatteman et al. 1995; Lindahl et al. 1997]. Analysis of PDGF-B and βPDGF null embryos suggests that PDGF-B released by ECs drives the proliferation and migration of βPDGF-positive VSMCs along angiogenic sprouts [Hellstrom et al. 1999]. In ShcA-deficient embryos, VSMCs fail to expand along endothelial tubes, indicating that ShcA is required for vessel wall development.

In addition to its role in angiogenesis, ShcA is highly expressed in the embryonic heart, and ShcA mutants have reduced cardiac trabeculation and a hypoplastic endocardial cushion. Development of the myocardium and endocardium requires signaling by soluble factors such as neuregulin and Ang-1, and the cell surface protein ephrin-B2 [for review, see Yancopoulos et al. 1998]. Targeted mutations that affect neuregulin [Meyer and Birchmeier 1995] or the myocardial RTKs ErbB2 [Lee et al. 1995] and ErbB4 [Gassmann et al. 1995] cause very similar phenotypes in the heart as the ShcA mutation. Mutations in EphB2/B3 [Adams et al. 1999], Ang-1 [Suri et al. 1996], and Tie-2/Tek [Dumont et al. 1994; Sato et al. 1995] also cause trabeculation defects resembling those in ShcA mutant embryos.

Taken together, these data suggest that ShcA is a target for one or more of the RTKs involved in cardiovascular development. Several of these receptors, including Flt4/VEGFR3 and ErbB2/4 have binding sites for the ShcA PTB domain [Fournier et al. 1995; Lamint et al. 1996], whereas others such as the βPDGF associate with the ShcA SH2 domain [Yokote et al. 1994]. Multiple ligand-receptor systems may therefore signal through ShcA to establish a mature vascular system.

**ShcA sensitizes cells to growth-factor signaling**

These observations raise the question of the biochemical functions of ShcA in the developing vasculature. ShcA has been implicated in activation of the Ras/MAPK pathway through its ability to bind the Grb2–Sos1 complex. By using mutant MEFs, we found that ShcA was dispensable for MAPK activation in response to moderate concentrations of EGF or PDGF, but was necessary for efficient MAPK activation in cells exposed to low amounts of growth factors. These results suggest that when the supply of growth factor is abundant, the ability of autophosphorylated EGF or PDGF receptors to bind Grb2 directly is sufficient to fully stimulate the MAPK pathway. However, at low concentrations of growth factors, the ShcA adaptor is required to sensitize the cellular machinery that activates the MAPK pathway. This interpretation is supported by the finding that low concentrations of EGF that fail to elicit binding of Grb2 to the receptor, nonetheless induce ShcA phosphorylation and formation of a ShcA–Grb2–Sos1 complex [Wennstrom and Downward 1999]. ShcA is generally considered to be broadly expressed. However, in situ hybridization analysis indicates that ShcA expression is, in fact, highly dynamic in vivo. Thus, the ability of cells to respond to external cues during embryogenesis may in part be determined by their level of ShcA expression. A signal that increased ShcA expression could then render cells permissive to respond to small increases in growth-factor stimulation that would otherwise fail to activate the MAPK pathway.

To test whether the ability of ShcA to potentiate signaling may underlie the defects in mutant embryos, we probed the state of MAPK activation in vivo using phosphospecific MAPK antibodies. Mutant embryos show a striking and specific loss of phosphorylated MAPK in the cardiovascular system that precede the onset of phenotypic abnormalities and coincide with the pattern of ShcA expression. Because other sites of MAPK activation, such as the limb bud, were not significantly affected in mutant embryos, these data argue that ShcA is not an invariant component of pathways leading to MAPK activation, but rather plays a specific role in coupling RTKs to such pathways in specific embryonic tissues such as the heart and blood vessels. The notion that tight control of the Ras/MAPK pathway is important in cardiovascular development receives support from the phenotype of mice lacking the p120-Ras GTPase activating protein [Henkemeyer et al. 1995]. Such embryos die by E10.5 with severe defects in angiogenesis, including a failure to remodel the yolk sac and thinning of the dorsal aorta.

These data suggest a functional distinction between the adaptor proteins that mediate pTyr signaling. A protein such as Grb2 is highly conserved in evolution, and is a component of a core pathway with an essentially invariant function in metazoan animals. In contrast, Shc proteins have apparently evolved as multicellular organisms became more complex, and may serve to amplify and coordinate the activities of core factors such as Grb2, in response to an increasing array of extracellular signals. Consistent with this view, genetic analysis in Dro sophila has demonstrated that dshc appears to mediate a specific aspect of signaling by a subset of RTKs, as dshc mutations cause a partial reduction in the functions of the Torso receptor and DER [Luschnig et al. 2000].

**ShcA regulates cellular morphology**

The proliferation and migration of vascular cells require...
integrins (Stromblad and Cheresh 1996). Previous work has suggested that ShcA is a binding partner and sub-
strate of integrin-activated tyrosine kinases such as FAK
and Src (Schlaepfer et al. 1999), and appears to contribute to
integrin-mediated MAPK activation (Wary et al. 1996;
Schlaepfer and Hunter 1997). We found that Erk activa-
tion was reduced in mutant cells plated on FN, indicat-
ing that ShcA provides a significant, but not a unique
route for integrin-activated kinases to recruit Grb2. Sur-
prisingly, ShcA mutant cells plated on FN have an aber-
rant rounded morphology, characterized by an increased
number of small focal complexes, and a disorganized,
depolarized actin cytoskeleton. This function of ShcA
may relate to some of the vascular defects in mutant
embryos, such as the failure of mesenchymal cells in the
dermal cushion to project processes that invade the
surrounding FN-enriched ECM.

Conclusion

ShcA has been viewed as a generic adaptor. In contrast,
the present data suggest that ShcA has highly specific
functions in mediating signals generated by complex
cell–cell interactions. ShcA sensitizes cells in culture
and in vivo for MAPK activation and controls cytoskel-
et architecture. These combined activities suggest how
ShcA may function during cardiovascular development.
Consistent with a more general role for ShcA in the
morphogenesis of complex structures, E10 ShcA mutant em-
byros show reduced branching of specific cranial ganglia
(unpubl.), and transgenic expression of ShcA in mouse
mammary epithelial cells induces increased tertiary
branching of the virgin mammary gland (Rauh et al.
1999).

Materials and methods

Construction of the ShcA targeting vector and generation
of chimeric mice

The ShcA targeting vector was constructed by inserting a 5.4-kb
ShcA genomic fragment from 129Sv strain DNA into the vector
pPNT. The 3′ arm, consisting of a 2.5-kb fragment, was inserted
between the neo8 and tk selection cassettes. NotI-linearized
targeting vector (30 µg) was electroporated into R1 ES cells as
described (Nagy et al. 1993; Wood et al. 1993). The frequency of
homologous recombination was 1 in 33 (n = 200). Aggregation
chimeras were generated with targeted ES cells and male chi-
meric mice were then bred with 129Sv or CD1 females to obtain
germ-line transmission. Timed matings were carried out by as-
signing the morning of identification of vaginal plugs as day 0.5
(E0.5).

Southern blotting and PCR

Genomic DNA prepared from tail biopsies or embryonic yolk
sac was subjected to PCR and/or Southern blot analysis. For
Southern blot analysis, 5 µg of genomic DNA was digested with
NcoI, separated on 0.6% agarose gels, and then blotted onto
nylon membrane filters (Genescreen, NEN Life Science). For
PCR reactions, tissues were digested with proteinase K over-
night at 55°C. The reaction was cycled 30 times (1 min at 94°C,
1 min at 56°C, 2 min at 72°C), which amplified a 410-bp frag-
ment of the wild-type ShcA gene and a 347-bp fragment of the
mutant allele. Oligos used were PCR-5′ (5′-TGCTCTAGCTCT-
TGCTCTGGTAG-3′) corresponding to the 5′ flanking region,
PCR-3′ (5′-GTTAAAGAAACTCTGGACCAATC-3′) matching
the deleted part of the DNA, and neo8 (5′-ATGCCCT-
GCTCTTTTACTGAAGGC-3′) corresponding to the neo8 gene
[Fig. 1A].

RNA preparation and Northern blot analysis

RNA was isolated from various tissues as described (Conlon and Rossant 1992). To make the RNA probe, a fragment
(corresponding nucleotides 1–1505 of p66 cDNA) from the
mouse ShcA CDNA in pBSSK vector was linearized with StuI
and transcribed with T7 RNA polymerase (sense probe), or
linearized with BamHI and transcribed with T7 RNA polymer-
ase (sense probe).

RNA in situ hybridization analysis

RNA in situ hybridization analysis was performed as described
(Henkemeyer et al. 1995). Embryos were then sectioned and counterstained with hematoxylin.

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization analysis was performed as described
(Colon and Rossant 1992). To make the RNA probe, a fragment
(corresponding nucleotides 1–1505 of p66 cDNA) from the
mouse ShcA CDNA in pBSSK vector was linearized with StuI
and transcribed with T3 RNA polymerase (anti-sense probe), or
linearized with BamHI and transcribed with T7 RNA polymer-
ase (sense probe).

Immunohistochemistry

Whole-mount embryo immunostaining with anti-CD-31 (PEC-
CAM-1, Pharmingen, 1:1000), anti-SMaA (clone 1A4, Sigma,
1:500) was performed as described (Henkemeyer et al. 1994).
DAB-stained embryos were postfixed, photographed, sectioned, and
counterstained with hematoxylin. For whole-mount immu-
nostaining with anti-dpERK (Sigma, 1:250), embryos were
frozen-sectioned, fixed in 8% paraformaldehyde for 4 hr to over-
night at 4°C, washed in PBS containing 0.5% NP-40, and im-
munostained with secondary antibodies of peroxidase-conjugated Affini-
Pure goat anti-mouse IgG (Jackson Labs, 1:250) and alkaline phos-
phatase-conjugated goat anti-rabbit IgG (H + L, mouse serum
pre-adsorbed, Bio-Rad, 1:400) were used for anti-dp-ERK and
anti-CD-31 staining, and were detected with DAB and BCIP,
respectively.

For immunohistochemical staining of sections with anti-
SMcA (Sigma, 1 µg/ml), 7–10 µm paraffin sections were blocked
with 10% normal goat serum and 0.15% Triton X-100 and in-
cubated with primary antibody overnight at 4°C in a humidified
chamber. The slides were washed at least three times in PBS and
then further processed using Vectastain ABC elite detection kit
(Vector laboratory).
Lysate preparation and immunoblotting

E9.5 or E10.5 embryos were homogenized in PLC-lysis buffer using a Teflon homogenizer essentially as described (Lai et al. 1995). Supernatants were collected after repeated centrifugation. For each immunoprecipitation, the equivalent of one E10.5 embryo (~2 mg protein) was used, followed by SDS-PAGE, and filters were probed with appropriate antibodies and developed using either an ECL kit, ECL + Plus kit or [125I]-protein A (Amersham).

Transmission electron micrographs

E10 embryos were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Ultrathin [50 nm] transverse plastic sections of genotyped embryos were stained with uranyl acetate and lead citrate and viewed at 60 kV in a Philips CM100 Biotwin TEM.

Generation of MEFs cell lines

Dissected E10 embryos were incubated with trypsin for 15 min at 37°C, then seeded on 6-cm² tissue culture plates in DMEM containing 15% FBS. Yolk sacs from each embryo were genotyped by PCR. Experiments described in this study were repeated with cell lines derived from separate embryos collected from different parents.

Expression of p52/46 ShcA in ShcA mutant MEFs

A 1.6-kb BglII–EcoRI mouse ShcA cDNA fragment (Migliaccio et al. 1997) encoding the p52/46 ShcA polypeptides was engineered into MSCVpac vector [Markowitz et al. 1988] at Xhol–EcoRI site. Subconfluent ShcA mutant MEFs were transfected overnight with Lipofectamine (GIBCO BRL) containing 15 µg of DNA in a 150-mm cell culture dish. Cells were first cultured in DMEM with 10% FBS for 24 hr and then selected with puromycin (Sigma, 1 µg/ml). Puromycin-resistant clones were isolated and screened for expression of ShcA proteins by immunoblotting using anti-ShcA antibody (Pelicci et al. 1992). Expression level was quantified using ECL + Plus (Amersham).

Characterization of MAPK activation

Subconfluent cells were serum starved for 2 days in DMEM containing 20 mM HEPEs [pH 7.2], then stimulated with EGF or PDGF-BB at 37°C. For studies with FN [Sigma, 10 µg/ml] or PLK (Sigma, 0.5 mg/ml), cells were treated essentially as described [Schlaepfer and Hunter 1997]. Stimulated cells were then lysed in 2× SDS-sample buffer and boiled. Equal amounts of lysates were resolved by SDS-PAGE and analyzed by immunoblotting with the anti-Erk2 monoclonal antibody [UBL, 0.5 µg/ml], anti-phospho-MAPK antibody [NEB, 1 µg/ml], or with anti-Erk1 monoclonal antibody (Santa Cruz, 0.5 µg/ml) as indicated.

Detection of focal contacts formation and actin organization

Quiescent cells were trypsinized and plated on FN-coated glass coverslips for 40 min at 37°C, washed, fixed for 15 min in 4% paraformaldehyde with 5% sucrose, then permeabilized with 0.5% Triton X-100 for 5 min, and finally blocked in PBS containing 5% BSA and 2% normal goat serum. Following incubation with anti-paxillin mAb [transduction, 1 µg/ml] or anti-vinculin mAb [Sigma, 1 µg/ml] overnight at 4°C, cells were washed and then treated with fluorescein goat anti-mouse IgG [Molecular Probes, 1:100] and Texas Red-X phalloidin [Molecular Probes, 1:100]. Cells were subsequently washed, labeled with Hoechst no. 33258 [Sigma, 1 µg/ml], mounted, and analyzed using a Leica DMRXE microscope equipped with appropriate filters and using 100× oil-immersion objectives.

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The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo

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