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TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA

Matt K Lee[1,*], Cécile Pardoux[2], Marie C Hall[2], Pierre S Lee[2], David Warburton[3], Jing Qing[2], Susan M Smith[1] and Rik Derynck[2]

[1]Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA, USA, [2]Programs in Cell Biology and Developmental Biology, Department of Cell and Tissue Biology and Anatomy, University of California, San Francisco, CA, USA and [3]Saban Research Institute, Childrens Hospital Los Angeles, University of Southern California, CA, USA

Erk1/Erk2 MAP kinases are key regulators of cell behaviour and their activation is generally associated with tyrosine kinase signalling. However, TGF-β stimulation also activates Erk MAP kinases through an undefined mechanism, albeit to a much lower level than receptor tyrosine kinase stimulation. We report that upon TGF-β stimulation, the activated TGF-β type I receptor (TβRI) recruits and directly phosphorylates ShcA proteins on tyrosine and serine. This dual phosphorylation results from an intrinsic TβRI tyrosine kinase activity that complements its well-defined serine-threonine kinase function. TGF-β-induced ShcA phosphorylation induces ShcA association with Grb2 and Sos, thereby initiating the well-characterised pathway linking receptor tyrosine kinases with Erk MAP kinases. We also found that TβRI is tyrosine phosphorylated in response to TGF-β. Thus, TβRI, like the TGF-β type II receptor, is a dual-specificity kinase. Recruitment of tyrosine kinase signalling pathways may account for aspects of TGF-β biology that are independent of Smad signalling.

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Introduction

Cell behaviour is regulated by growth factors that activate transmembrane receptor kinases. The substrates of these kinases initiate enzymatic cascades that ultimately regulate gene transcription. Although growth factors associate with specific receptors, the pathways initiated by these kinases are highly interconnected. Entire classes of receptors phosphorylate identical substrates, whereas multiple pathways converge on key intracellular effectors. Prominent among these effectors are the Erk1/Erk2 MAP kinases. Erk1 and Erk2 phosphorylate an array of transcription factors, thereby regulating cell proliferation, apoptosis and differentiation (Qi and Elion, 2005). MAP kinases represent the final cytoplasmic components of signalling pathways initiated by receptor tyrosine kinases and G protein-coupled receptors.

Shc adaptor proteins are substrates of receptor tyrosine kinases, and their phosphorylation initiates signalling events that culminate in Erk activation (Ravichandran, 2001). Among the three related Shc proteins, ShcA is ubiquitously expressed, whereas ShcB and ShcC are restricted to cells of neural origin. ShcA, in turn, is expressed as three isoforms. The prototype $p52^{ShcA}$ consists of an N-terminal PTB domain followed by CH1 and SH2 domains. $p66^{ShcA}$ is identical to $p52^{ShcA}$ except for an added N-terminal CH2 domain, whereas $p46^{ShcA}$ results from N-terminal truncation of the $p52^{ShcA}$ PTB domain. The PTB and SH2 domains both bind tyrosine-phosphorylated peptides, and either may associate with activated receptor kinases (Kavanaugh and Williams, 1994). Tyrosine phosphorylation enables $p52^{ShcA}$ to bind the Grb2 adaptor and the Sos GTP-exchange factor (van der Geer *et al*, 1996). The ShcA/Grb2/Sos complex converts Ras into its active GTP-bound form, leading to the sequential activation of c-Raf, MEK and Erk1/Erk2. In contrast, $p66^{ShcA}$ antagonises Erk activation, possibly by sequestering Grb2 (Migliaccio *et al*, 1997), and mediates an oxidative stress signalling function (Migliaccio *et al*, 1999). $p46^{ShcA}$ is targeted to mitochondria (Ventura *et al*, 2004), where its role is unclear.

TGF-β stimulation also activates Erk MAP kinases, albeit to a much lower level than receptor tyrosine kinases (Mulder, 2000). TGF-β proteins are key regulators of development and tissue differentiation (Attisano and Wrana, 2002), and signal through complexes of type II (TβRII) and type I (TβRI) receptors that are characterised as serine-threonine kinases (Chen *et al*, 1995). However, TβRI and TβRII kinase domains share homologies with tyrosine kinases (Manning *et al*, 2002), and TβRII autophosphorylates on tyrosine as well as on serine and threonine (Lawler *et al*, 1997). Engagement of the receptor complex activates TβRI, which phosphorylates and activates Smad2 and Smad3 (Derynck and Zhang, 2003). These proteins then complex with Smad4, translocate to the nucleus, and associate with DNA-binding complexes to regulate gene transcription.

The Smad pathway does not explain Erk activation by TGF-β. The kinetics of this process vary with cell type and culture conditions (Massagué, 2000). In some cell lines, delayed activation suggests an indirect response requiring protein translation (Simeone *et al*, 2001), whereas in others, activation is rapid and comparable to signalling by mitogenic factors such as EGF (Olsson *et al*, 2001). Although TGF-β induces modest Ras activation consistent with low level Erk induction (Mulder, 2000), the mechanisms underlying this induction are unclear.

Despite its low level, Erk activation is important to TGF-β signalling. First, Erk activation and Smad signalling are both necessary for TGF-β-induced epithelial-mesenchymal

*Corresponding author. Center for Craniofacial Molecular Biology, University of Southern California, 2250 Alcazar Street, CSA-103, Los Angeles, CA 90033, USA. Tel.: +1 323 442 3805; Fax: +1 323 442 2981; E-mail: mattlee@usc.edu

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transformation (Davies *et al*, 2005), a key event in neoplastic invasion and metastasis. Second, Erk MAP kinases phosphorylate receptor-activated Smads to regulate their nuclear translocation (Kretzschmar *et al*, 1999). Finally, Erk substrates interact with Smads to regulate gene expression (Mucsi *et al*, 1996). Thus, the mechanism by which TGF-β activates Erk MAP kinases is an unresolved issue of considerable interest (Massagué, 2000). We now report that upon TGF-β stimulation, TβRI phosphorylates ShcA on serine and, to a lesser degree, on tyrosine to activate Erk MAP kinases.

Results

TGF-β rapidly induces serine and tyrosine phosphorylation of ShcA

TGF-β-induced ShcA tyrosine phosphorylation was assessed in Mv1Lu mink epithelial cells and 3T3-Swiss mouse fibroblasts using anti-phosphotyrosine antibodies. TGF-β stimulated rapid tyrosine phosphorylation of all three isoforms that peaked after 5–20 min (Figure 1A and B; quantified in Supplementary Figure 1). The tyrosine phosphorylation of ShcA induced by TGF-β was generally higher in 3T3-Swiss than in Mv1Lu cells, but much lower than that induced by EGF (data not shown; Figure 2A). The rapid ShcA phosphorylation suggested that new gene expression was not required. This was confirmed by persistent TGF-β-induced ShcA tyrosine phosphorylation in the presence of the protein synthesis inhibitor cycloheximide (Figure 1C).

TGF-β-induced ShcA phosphorylation *in vivo* was characterised by phosphoamino acid analysis. ^{32}P-labelled ShcA was immunoprecipitated, isolated by SDS–PAGE (Figure 2A), and acid hydrolysed. The labelled amino acids were then resolved by two-dimensional electrophoresis. For all three ShcA isoforms, serine and, to a much lesser extent, tyrosine phosphorylation increased rapidly after TGF-β stimulation (Figure 2B–D). In contrast, only tyrosine was phosphorylated by EGF stimulation.

Roles of type II and type I receptors in TGF-β-induced ShcA phosphorylation

TGF-β binding induces TβRII to phosphorylate TβRI, which in turn phosphorylates Smad proteins. To address the role of TβRII in TGF-β-induced ShcA phosphorylation, we expressed tagged ShcA in Mv1Lu cells together with a cytoplasmic truncation of TβRII, a kinase-deficient point mutant of TβRII, or a control vector. TGF-β-induced ShcA tyrosine phosphorylation was abrogated by truncated (Figure 3A) or kinase-deficient TβRII (data not shown), indicating that TβRII signalling is essential for this response.

We also treated 3T3-Swiss cells with SB431542, a specific inhibitor of TβRI (Inman *et al*, 2002). SB431542 treatment prevented TGF-β from inducing ShcA tyrosine phosphorylation (Figure 3B; quantified in Supplementary Figure 2). Together, these data indicate that upon activation by TβRII, TβRI induces ShcA phosphorylation.

ShcA proteins interact with TGF-β receptors

Rapid ShcA phosphorylation following TGF-β stimulation led us to hypothesise that ShcA proteins physically interact with TGF-β receptors. To evaluate this postulate, COS cells were transfected to coexpress p66^{ShcA} and p52^{ShcA} with TβRII or TβRI. p46^{ShcA} was not evaluated as its role in signalling is unclear. As shown in Figure 4A, p66^{ShcA} and p52^{ShcA} co-precipitated with TβRII and TβRI, with p66^{ShcA} co-precipitating less efficiently than p52^{ShcA}. As p66^{ShcA} differs from p52^{ShcA} only in the addition of an N-terminal CH2 domain, we conclude that this domain is not required for receptor interaction and may decrease interaction efficiency.

We also assessed whether ShcA interacts with TGF-β receptors at the cell surface. 3T3-Swiss cells expressing TβRII with or without coexpressed TβRI were incubated with ^{125}I-TGF-β1. The radiolabelled ligand was then chemically crosslinked to its receptors, and endogenous ShcA

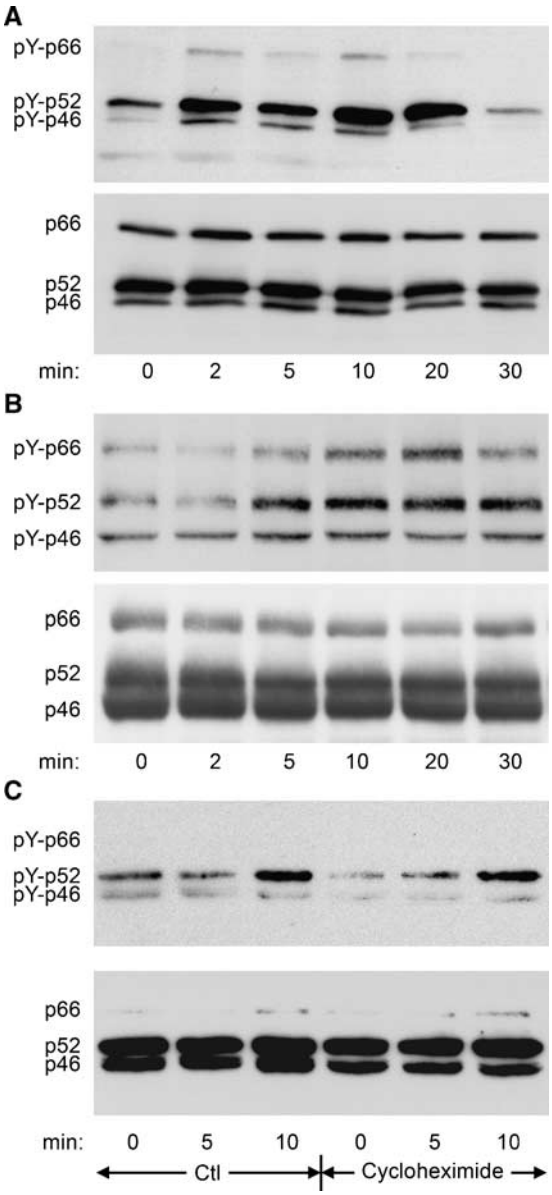


Figure 1 TGF-β induces ShcA tyrosine phosphorylation. (**A, B**) Anti-phosphotyrosine Western blot of ShcA immunoprecipitated from lysates of Mv1Lu cells (A) or 3T3-Swiss cells (B) treated with 4 ng/ml TGF-β for the indicated times (upper panels), together with ShcA immunoblots of the same membranes (lower panels). (**C**) Anti-phosphotyrosine Western blot of ShcA immunoprecipitated from lysed 3T3-Swiss cells treated with or without cycloheximide before and concomitant with stimulation with 4 ng/ml TGF-β for the indicated times. The same membrane was reprobed for ShcA (lower panel).

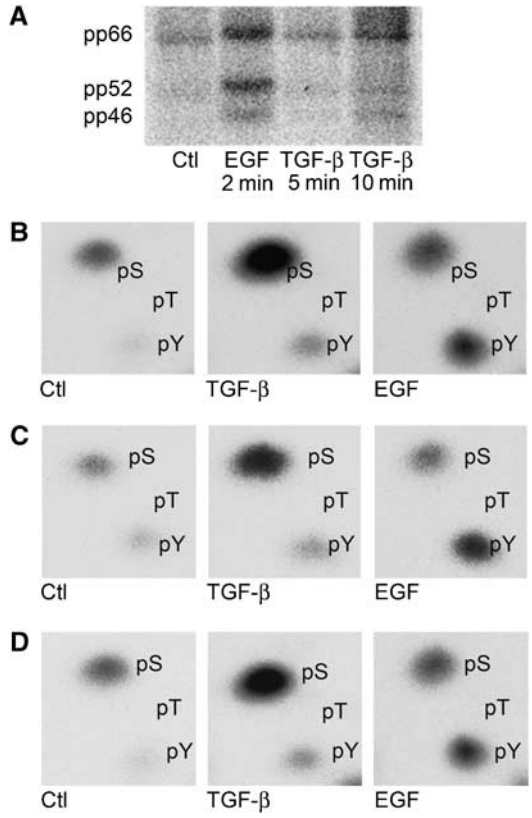


Figure 2 TGF-β induces ShcA phosphorylation on serine and tyrosine. (**A**) Autoradiogram of 3T3-Swiss cells cultured in the presence of ^{32}P-[PO_4] and treated with 4 ng/ml TGF-β, 20 ng/ml EGF, or neither (Ctl) for the indicated times. (**B**–**D**) Phosphoamino acid analysis of *in vivo* ^{32}P-phosphorylated p66^{ShcA} (B), p52^{ShcA} (C), or p46^{ShcA} (D) isolated from the membrane shown in (A). The ^{32}P-labelled amino acids migrated in the same positions as unlabelled phosphoserine and phosphotyrosine added to the reaction mixture. No ^{32}P-labelled phosphothreonine was detected.

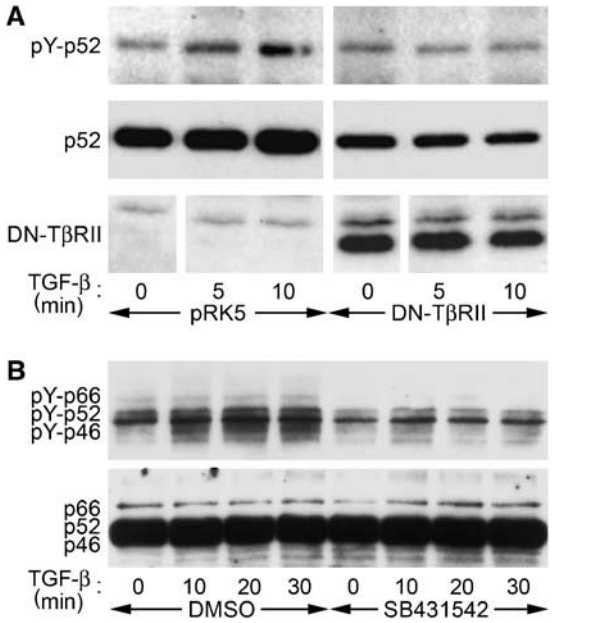


Figure 3 TβRII and TβRI receptors are required for TGF-β-induced ShcA tyrosine phosphorylation. (**A**) Anti-phosphotyrosine Western blot of HA-tagged p52^{ShcA} immunoprecipitated from Mv1Lu cells that do or do not coexpress a cytoplasmically truncated, dominant-negative version of TβRII. (**B**) Anti-phosphotyrosine blot of ShcA immunoprecipitated from 3T3-Swiss cells treated with 4 ng/ml TGF-β for the indicated times after 30 min pretreatment with 10 μM TβRI inhibitr SB431542 or DMSO solvent.

was immunoprecipitated. ^{125}I-TGF-β1 binds TβRII or TβRII in complex with TβRI (TβRII/RI), but not TβRI alone (Yamashita *et al*, 1994). Under these conditions, ^{125}I-TGF-β1 also crosslinks to TβRIII. As shown in Figure 4B, ^{125}I-TGF-β-labelled TβRII interacted with endogenous ShcA. Increased expression of p66^{ShcA} (or p52^{ShcA}; data not shown) enhanced the co-precipitation of TβRII alone or TβRI co-expressed with TβRII, whereas the total level of ^{125}I-TGF-β-labelled receptors remained unaffected (Figure 4B, lanes 2 and 4). Because ^{125}I-TGF-β1 co-precipitated with ShcA more efficiently when TβRI was coexpressed (Figure 4B, lane 4 versus lane 3), we suggest that ShcA interacts more efficiently with the TβRII/RI complex than with TβRII alone.

In nontransfected 3T3-Swiss cells, the interaction between endogenous ShcA and TβRII or TβRI was greatly enhanced by TGF-β stimulation (Figure 4C and D). This association was maximal at 10 min, coincident with peak TGF-β-induced ShcA phosphorylation. The low level interaction of ShcA with TβRI in the absence of exogenous TGF-β likely reflects autocrine TGF-β signalling, which is common in cultured cells (Derynck *et al*, 2001).

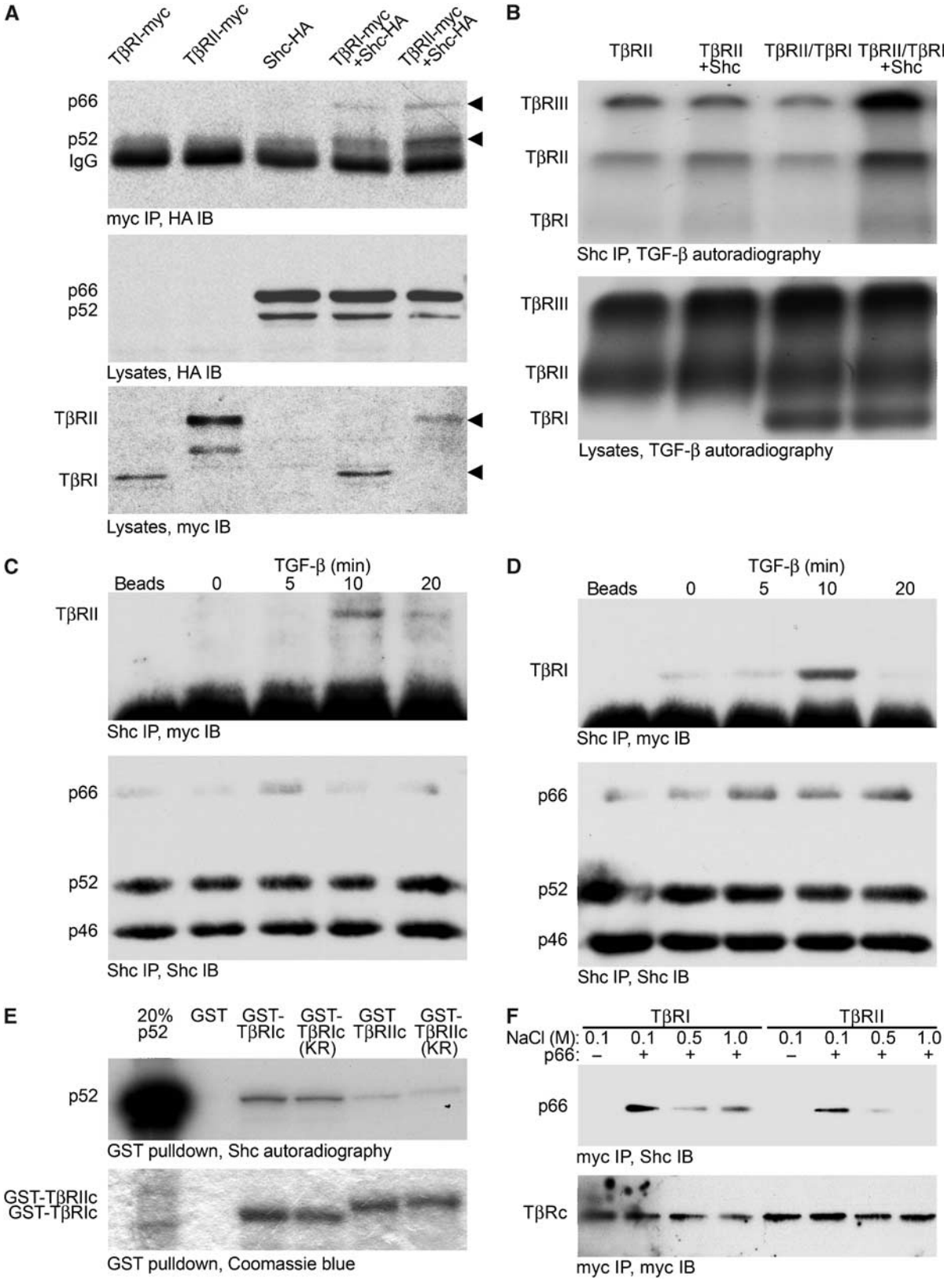
The preferential interaction of ShcA with TβRI was also assessed *in vitro*. In these assays, *in vitro* translated, ^{35}S-labelled p52^{ShcA} interacted more efficiently with wild-type or kinase-deficient glutathione-*S*-transferase (GST)-fused TβRI cytoplasmic domains than with TβRII cytoplasmic domains (Figure 4E). The interactions of p52^{ShcA} with the cytoplasmic domains of TβRI or TβRII *in vitro* were attenuated by increasingly stringent NaCl concentrations. Again, the interaction of ShcA with TβRI was more stable than with TβRII (Figure 4F). These results suggest that ShcA associates specifically and directly with TGF-β receptor cytoplasmic domains, and more strongly with TβRI than with TβRII.

p66^{ShcA} and p52^{ShcA} share a PTB domain, a CH1 domain, and an SH2 domain (Migliaccio *et al*, 1997). To determine which domains interact with TGF-β receptors *in vivo*, we evaluated several ShcA truncations. Deletion of the PTB domain abolished the interaction, whereas deletion of the SH2 domain did not detectably alter the interaction of ShcA with TβRI or TβRII (Figure 5). The CH1 domain alone did not interact with either receptor, whereas the PTB domain alone interacted efficiently with both. This association of the PTB domain with TβRI was confirmed *in vitro* (Supplementary Figure 3). We conclude that the PTB domain, present in all ShcA isoforms, is required for efficient association with TGF-β receptors.

TGF-β receptors directly phosphorylate ShcA

The physical association of ShcA with TGF-β receptors (Figures 3 and 4) and the rapidity with which TGF-β induced ShcA phosphorylation despite translational inhibition (Figure 1) suggested that activated TGF-β receptors directly phosphorylate ShcA. This function may be assigned to TβRI, as TGF-β-induced ShcA phosphorylation was inhibited by SB431542 (Figure 3B). Phosphorylation of ShcA on serine and tyrosine by TβRI *in vitro* would indicate that TβRI, like TβRII (Lawler *et al*, 1997), is a dual-specificity kinase.

To assess this possibility, we expressed the TβRI and TβRII cytoplasmic domains in *Escherichia coli* as GST fusion proteins and immobilised the purified proteins to glutathione-Sepharose. Incubation of purified $p52^{ShcA}$ or $p66^{ShcA}$ (also made in *E. coli*) with TβRI or TβRII kinase in the presence of γ-^{32}P-ATP resulted in ShcA phosphorylation *in vitro* (Figure 6A; $p66^{ShcA}$ data not shown). Phosphoamino acid analysis revealed that both kinases phosphorylated $p52^{ShcA}$ (Figure 6B and C) and $p66^{ShcA}$ (data not shown) predominantly on serine, with low levels of tyrosine and threonine



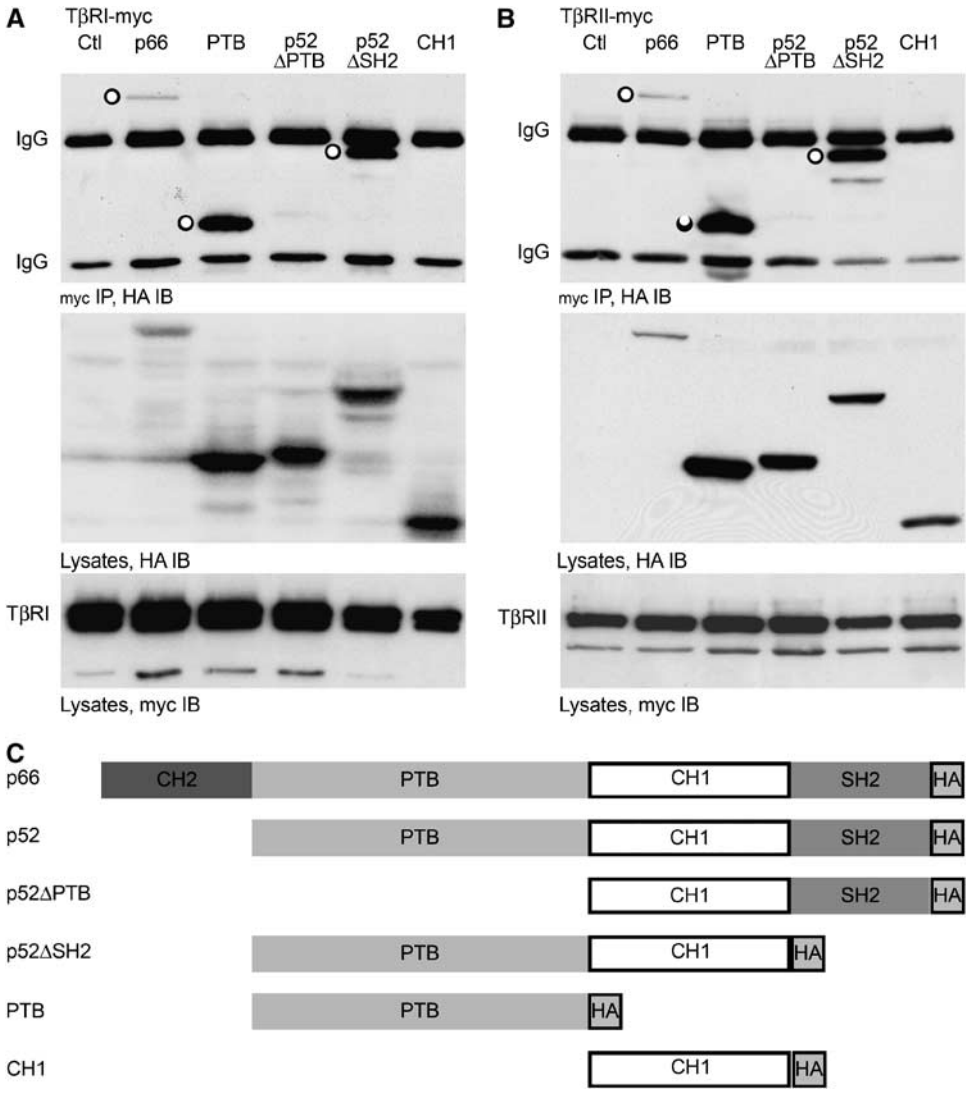


Figure 5 ShcA associates with TGF-β receptors through its PTB domain. $p66^{ShcA}$ differs from $p52^{ShcA}$ only by an N-terminal addition of a CH2 domain; otherwise, both $p66^{ShcA}$ and $p52^{ShcA}$ are comprised of PTB and SH2 phosphotyrosine-binding domains flanking a central CH1 domain. HA-tagged $p66^{ShcA}$, ShcA fragments, or $p52^{ShcA}$ deficient in the SH2 domain (ΔSH2) or the PTB domain (ΔPTB) were expressed in 293 cells together with myc-tagged cytoplasmic domains of TβRI or TβRII. (**A**, **B**) HA Western blot of myc immunoprecipitates (top), HA immunoblot (centre), and myc immunoblot (bottom) of cells co-transfected with TβRI-myc (A) or TβRII-myc (B) and ShcA constructs. '○' indicates ShcA construct co-precipitated with TGF-β receptor. (**C**) Schematic representation of ShcA expression constructs used.

Figure 4 ShcA interacts with TGF-β receptors. (**A**) $p66^{ShcA}$ and $p52^{ShcA}$ co-precipitate with TGF-β receptors in COS cells. Cells expressing myc-tagged TβRI or TβRII, and/or HA-tagged $p66^{ShcA}$ and $p52^{ShcA}$, as indicated, were subjected to myc immunoprecipitation followed by HA immunoblotting (top). Expression of ShcA and TGF-β receptors was monitored by HA (middle) or myc (bottom) Western analysis of cell lysates. (**B**) ShcA interacts with cell surface TGF-β receptors. 3T3-Swiss cells were transfected to express TβRII and/or TβRI with or without $p66^{ShcA}$, and cell surface receptors were radiolabelled with ^{125}I-TGF-β1 followed by chemical crosslinking. Immunoprecipitation of both endogenous and transfected ShcA followed by autoradiography (top panel) demonstrated co-precipitation of ^{125}I-labelled TGF-β receptors with ShcA. ^{125}I-labelled TGF-β receptors did not precipitate when the ShcA antibody was replaced with non-immune IgG (data not shown). Autoradiography of total cell lysates (below) showed equivalent levels of ^{125}I-labelled TGF-β receptors. (**C**, **D**) Ligand-dependent interaction of endogenous ShcA and TGF-β receptors in mammalian cells. 3T3-Swiss cells were treated with 4 ng/ml TGF-β for the indicated times, lysed, and subjected to ShcA immunoprecipitation followed by TβRII (C) or TβRI (D) immunoblotting. Control precipitations without primary antibody are shown ('beads'). Subsequent ShcA immunoblots (lower panels) confirmed equivalent precipitation efficiencies. (**E**) *In vitro* association of $p52^{ShcA}$ with TβRI and TβRII cytoplasmic domains. *In vitro* translated ^{35}S-labelled $p52^{ShcA}$ was incubated with GST, GST-fused TβRI or TβRII cytoplasmic domains, or their kinase-deficient point mutants (TβRIc, TβRIIc, TβRIc KR, and TβRIIc KR, respectively). ^{35}S-labelled ShcA adsorbed to the GST fusion proteins was visualised by SDS–PAGE and autoradiography (upper panel). To confirm identity, ^{35}S-labelled $p52^{ShcA}$ was loaded in lane 1 at 20% of the volume used in the binding assay. Equivalent GST fusion protein loading was confirmed by Coomassie blue staining. (**F**) ShcA association with TβRI and TβRII cytoplasmic domains *in vitro* depends on NaCl concentration. Myc-tagged proteins incorporating either TβRIc or TβRIIc were expressed in COS cells, isolated by myc immunoprecipitation, and bound to recombinant $p66^{ShcA}$. The complexes were washed in otherwise identical buffers containing the indicated NaCl concentrations and subjected to ShcA immunoblotting (above). Myc Western analysis of the blot confirmed equivalent recovery of TβRIc and TβRIIc (below).

phosphorylation. The level of tyrosine relative to serine phosphorylation was much lower *in vitro* than *in vivo*, and the ShcA threonine phosphorylation observed *in vitro* was absent in TGF-β-stimulated cells (compare Figure 6B and C with Figure 2B and C). Lysates from untransformed *E. coli* did not phosphorylate ShcA (data not shown). As each reaction contained only purified kinase and substrate proteins, these results indicate that TβRI and TβRII function as dual specificity kinases of ShcA. The reduced tyrosine kinase activities of the TGF-β receptors in these experiments suggest, not surprisingly, that the conditions *in vitro* did not fully replicate conditions *in vivo*.

We also examined the phosphorylation of purified ShcA by a constitutively active chimera of the TβRII and TβRI cytoplasmic domains (RII-RI) that mimics the activated TβRII/TβRI complex *in vivo* (Feng and Derynck, 1996). Myc-tagged RII-RI chimeras comprised of wild-type kinases or chimeras in which one or both kinases were inactivated by point mutations were expressed from baculoviruses in insect cells and purified by affinity chromatography. As shown in Figure 6D, the chimera composed of two wild-type kinases (wtRII-wtRI) phosphorylated both itself and purified $p66^{ShcA}$ produced in *E. coli*. In contrast, the chimera of wild-type TβRII kinase fused to an inactive TβRI kinase (wtRII-RIKR) failed to phosphorylate $p66^{ShcA}$, but phosphorylated itself *in vitro* similar to wtRII-wtRI. The chimera of inactivated TβRII kinase fused to wild-type TβRI kinase and the chimera of two inactive kinases showed minimal autophosphorylation and did not phosphorylate ShcA (Figure 6D). These data indicate that the TβRI kinase phosphorylates ShcA, and that TβRII mediates the RII-RI autophosphorylation. Phosphoamino acid analysis of $p66^{ShcA}$ phosphorylated by wtRII-wtRI chimera *in vitro* again revealed predominant serine phosphorylation and much lower levels of tyrosine and threonine phosphorylation (Figure 6E). These data suggest that TβRI, like TβRII (Lawler *et al*, 1997), possesses intrinsic tyrosine kinase activity and that ShcA phosphorylation by TβRI *in vitro* requires TβRII kinase activity.

As ShcA associated with the TGF-β receptor complex following TGF-β stimulation (Figure 4D) through its phosphotyrosine-binding PTB domain (Figure 5; Supplementary Figure 3), we postulated that TβRI is tyrosine phosphorylated upon TGF-β binding. Anti-phosphotyrosine Western analysis showed that TβRI immunoprecipitated from transfected COS cells was tyrosine phosphorylated following *in vitro* kinase reactions (Figure 6F). To confirm this finding, Mv1Lu cells were stimulated with TGF-β for 10 min, and TβRI phosphorylation was evaluated by phosphotyrosine immunoprecipitation and TβRI immunoblotting. TβRI was rapidly tyrosine phosphorylated after TGF-β stimulation (Figure 6G). As TβRI is phosphorylated by TβRII, it is unclear whether this tyrosine phosphorylation results from TβRI autophosphorylation or from TβRII dual-specificity kinase activity (Lawler *et al*, 1997).

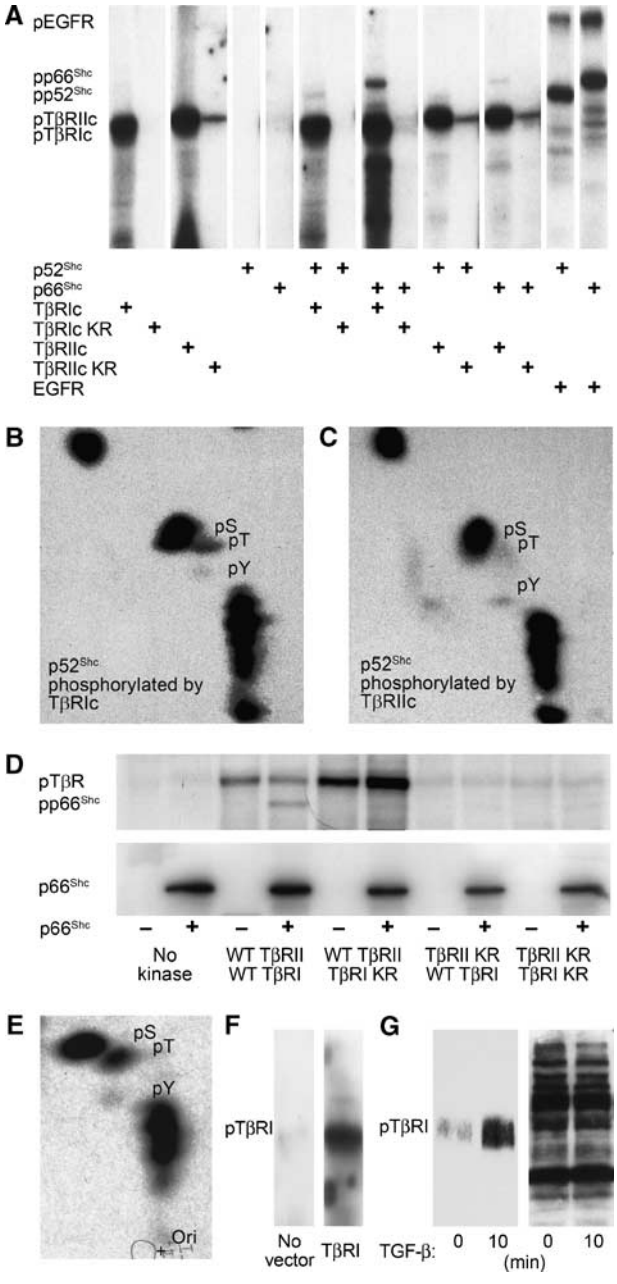


Figure 6 TGF-β receptors directly phosphorylate ShcA. (**A**) ShcA phosphorylation by purified GST-fused TβRI or TβRII cytoplasmic domains *in vitro*. Recombinant $p52^{ShcA}$ or $p66^{ShcA}$ was incubated with GST-fused wild type and/or kinase-inactive mutants (TβRIc, TβRIc KR, TβRIIc, or TβRIIc KR) or human EGF receptor in the presence of γ-^{32}P-ATP. The radiolabelled, autophosphorylated cytoplasmic domains, and ShcA proteins are indicated. (**B**, **C**) Phosphoamino acid analyses of $p52^{ShcA}$ phosphorylated by TβRI (B) or TβRII (C) cytoplasmic domains, as shown in panel (A). The locations of radiolabelled phosphoserine (pS), phosphotyrosine (pY), and phosphothreonine (pT) correlated with those of unlabelled phosphoamino acids added to the reaction mixture. (**D**) *In vitro* phosphorylation of *E. coli*-derived $p66^{ShcA}$ by TβRII-TβRI cytoplasmic chimeras generated in insect cells. Wild-type (WT) or kinase-inactivated (KR) receptor cytoplasmic domains were incubated with or without $p66^{ShcA}$. The radiolabelled $p66^{ShcA}$ and autophosphorylated RII-RI chimeras are visualised in the upper panel. ShcA immunoblotting confirmed equivalent $p66^{ShcA}$ levels in the reaction mixtures (lower panel). (**E**) Phosphoamino acid analyses of $p66^{ShcA}$ phosphorylated by wtRII-wtRI, as shown in panel (D). (**F**) Phosphotyrosine immunoblot of *in vitro* kinase reaction products containing myc immunoprecipitates from COS cells transfected with control pRK5 plasmid or pRK5 encoding myc-tagged TβRI. (**G**) TGF-β induces tyrosine phosphorylation of endogenous TβRI, as assessed by TβRI immunoblot of anti-pTyr immunoprecipitates from Mv1Lu cells treated with TGF-β for 10 min (left). Equal precipitation efficiency was demonstrated by phosphotyrosine immunoblotting of the precipitates (right).

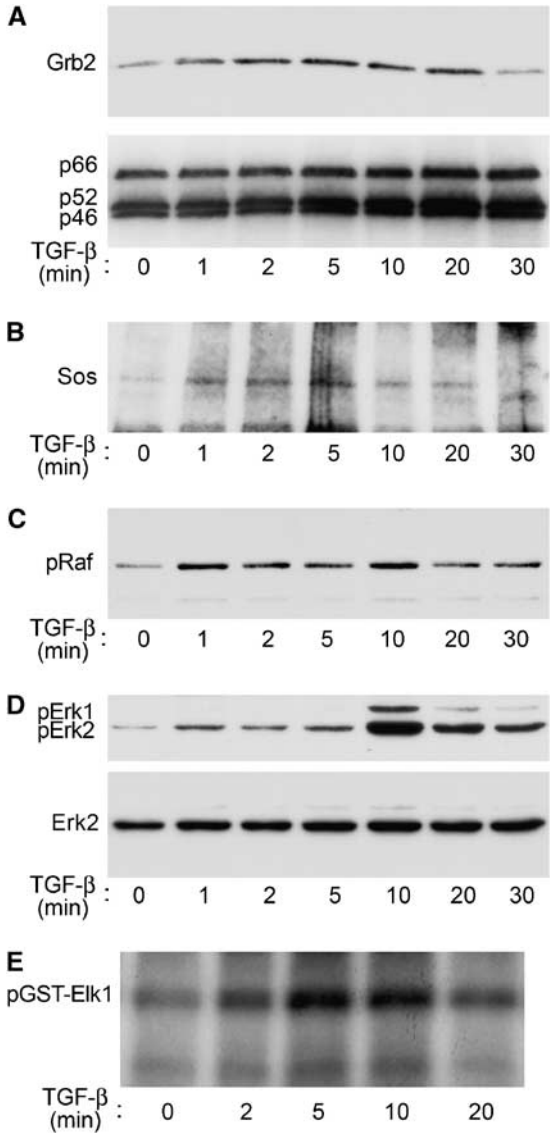


Figure 7 TGF-β activates the ShcA pathway in 3T3-Swiss cells. (**A**) TGF-β induces ShcA to associate with Grb2. Cells were stimulated with TGF-β for the indicated times, lysed, and subjected to ShcA immunoprecipitation followed by Grb2 Western analysis (upper panel). Reprobing the same membrane for ShcA confirmed equivalent precipitation efficiency (lower panel). (**B**) TGF-β induces ShcA to associate with Sos. The upper portion of the membrane shown in (A) was immunoblotted using an anti-Sos antibody. (**C**) TGF-β induces c-Raf phosphorylation, as assessed by phosphoRaf immunoblotting of stimulated cell lysates. (**D**) TGF-β induces Erk1/2 phosphorylation. The membrane in (C) was immunoblotted using a phosphoErk1/2-specific antibody (above). Reprobing the blot for Erk2 showed equivalent Erk expression and loading (below). (**E**) TGF-β activates Erk1/2. Stimulated cell lysates were incubated *in vitro* with Elk1-GST and γ^{32}P-ATP; ^{32}P-labelled Elk1-GST was visualised by autoradiography.

TGF-β induces signalling downstream of ShcA activation

In canonical ShcA signalling, receptor tyrosine kinases phosphorylate p52^{ShcA} on tyrosine to induce its association with Grb2 and Sos. This heterotrimer sequentially activates Ras, Raf, MEK1/2 and the Erk1/2 MAP kinases (Pelicci *et al*, 1992). Although TGF-β induces ShcA phosphorylation on serine as well as on tyrosine, our observations suggest that TGF-β activates the ShcA pathway. Low levels of ShcA tyrosine phosphorylation following TGF-β stimulation (relative to EGF) led us to predict that TGF-β activates the ShcA pathway much less efficiently than EGF.

We evaluated whether TGF-β stimulation enabled ShcA to bind Grb2 and Sos by stimulating 3T3-Swiss cells with TGF-β and immunoprecipitating the endogenous ShcA at defined times. The ShcA immunoprecipitates were then assessed for Grb2 and Sos association by Western analysis. As apparent in Figure 7A and B (and quantified in Supplementary Figure 4), TGF-β induced ShcA to associate with Grb2 and Sos with kinetics similar to TGF-β-induced ShcA phosphorylation (Figure 1). The amounts of Grb2 or Sos associated with ShcA were considerably less than in response to EGF (data not shown).

ShcA activation by receptor tyrosine kinases results in rapid phosphorylation of Raf. We therefore examined the effect of TGF-β on Raf phosphorylation in 3T3-Swiss cells using antibodies specific for phosphorylated Raf. As shown in Figure 7C, TGF-β induced rapid phosphorylation of Raf.

Finally, we evaluated the phosphorylation and activation of Erk1/2 MAP kinases. 3T3-Swiss cells were stimulated with TGF-β, and Erk1/2 phosphorylation was assessed using phospho-Erk-specific antibodies. TGF-β induced rapid Erk phosphorylation that peaked 5–10 min after addition of TGF-β (Figure 7D). This was similar to the kinetics of ShcA phosphorylation following TGF-β stimulation (Figure 1). Erk MAP kinase activation was also assessed by an Erk-specific *in vitro* kinase assay using immobilised Elk1 fused to GST as substrate (Figure 7E). In this assay, Erk kinase activity peaked 5–10 min after exposure to TGF-β, consistent with TGF-β-induced Erk phosphorylation. Together, these data suggest that TGF-β induces events immediately downstream of ShcA activation.

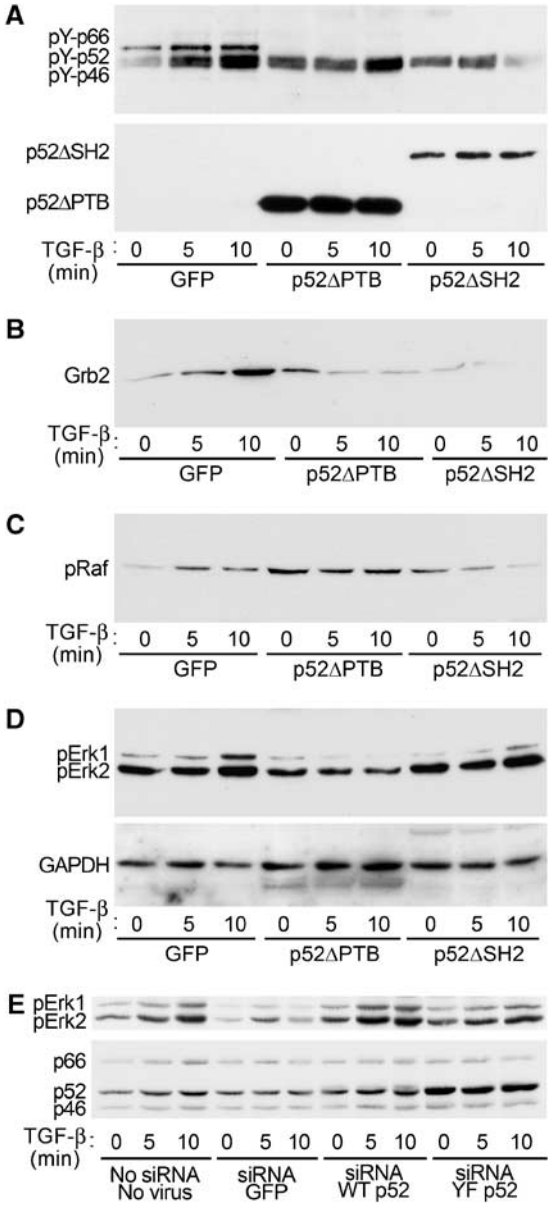
Direct induction of MAP kinase by TGF-β is mediated by ShcA

As TGF-β activates the MAP kinase pathway downstream of ShcA, we hypothesised that abrogating ShcA function would attenuate TGF-β-induced Erk activation. We overexpressed truncation mutants of ShcA that are expected to confer dominant inhibition of ShcB and ShcC as well as ShcA (Ravichandran, 2001). This approach was prompted by our observation that ShcA inactivation resulted in compensatory upregulation of ShcB and ShcC (data not shown), which may explain why mitogen-induced signalling is only modestly decreased in ShcA-deficient fibroblasts (Lai and Pawson, 2000). As ShcA binds TβRI through its PTB domain, we evaluated a PTB-deficient p52^{ShcA} mutant (p52^{ShcA}ΔPTB). This construct, which retains its Grb2 docking sites (van der Geer *et al*, 1996), does not associate with TβRI (Figure 5), co-precipitates with wild-type ShcA, and prevents TβRI from binding wild-type ShcA (Supplementary Figure 5). We also examined an SH2-deficient p52^{ShcA} mutant (p52^{ShcA}ΔSH2). Although the interaction of ShcA with the EGF receptor, like TβRI, depends on its PTB domain, the SH2 domain is required for EGF receptor-mediated ShcA phosphorylation (Gotoh *et al*, 1995). The mechanism for this requirement is uncertain.

As shown in Figure 8A (and quantified in Supplementary Figure 6), expression of p52^{ShcA}ΔPTB or p52^{ShcA}ΔSH2 in 3T3-Swiss cells attenuated TGF-β-induced ShcA tyrosine phosphorylation, suggesting dominant-negative interference.

Consistent with these observations, $p52^{ShcA}\Delta PTB$ or $p52^{ShcA}\Delta SH2$ expression reduced TGF-β-induced Grb2 recruitment to ShcA (Figure 8B) and Raf phosphorylation (Figure 8C). In the latter experiments, basal Raf phosphorylation was increased for unknown reasons. Finally, TGF-β-induced Erk1/2 phosphorylation was decreased in the presence of either $p52^{ShcA}$ mutant, with $p52^{ShcA}\Delta PTB$ being more effective than $p52^{ShcA}\Delta SH2$ (Figure 8D). Attenuation of TGF-β-induced Erk1/2 phosphorylation by $p52^{ShcA}\Delta PTB$ was observed in both 3T3-Swiss (Figure 8D) and Mv1Lu (data not shown) cells. We conclude that dominant-negative mutants of $p52^{ShcA}$ interfere with TGF-β responses downstream of ShcA activation.

To confirm that ShcA is required for TGF-β-induced Erk activation, 3T3-Swiss cells were transfected with an siRNA that silences all three ShcA isoforms (Kisielow *et al*, 2002). Cells were concomitantly infected with adenoviruses encoding wild-type $p52^{ShcA}$ or green fluorescent protein. In addition, we generated a vector encoding a mutant $p52^{ShcA}$ with tyrosines 239, 240, and 317 replaced by phenylalanines. These amino acids are phosphorylated by tyrosine kinases to become Grb2 binding sites, and their ablation prevents Grb2 binding (van der Geer *et al*, 1996). The $p52^{ShcA}$ expression vectors also incorporated a silent four-base mutation within the siRNA target sequence that conferred resistance to the siRNA. Transfection of ShcA siRNA resulted in partial ShcA downregulation that was nonetheless sufficient to abrogate TGF-β-induced Erk activation (Figure 8E). This abrogation was reversed by expression of wild-type, but not Grb2 binding-deficient $p52^{ShcA}$ (Figure 8E). These data confirm that ShcA is necessary for TGF-β-induced Erk1/2 activation, and implicate the same ShcA tyrosines that mediate receptor tyrosine kinase signalling.



Discussion

These data suggest that upon activation, TβRI directly phosphorylates ShcA on serine and tyrosine to induce its association with Grb2/Sos, thus initiating the pathway known to link receptor tyrosine kinases to Erk MAP kinases. Although phosphorylated by both TβRI and TβRII *in vitro*, ShcA interacted with and was phosphorylated by TβRI more efficiently than TβRII. *In vivo*, TGF-β-induced ShcA phosphorylation was abrogated by the TβRI inhibitor SB431542. Finally, a chimera comprised of an inactive TβRI kinase and a functional TβRII kinase abolished ShcA phosphorylation *in vitro* without affecting chimera autophosphorylation. The requirement of a functional TβRII for ShcA phosphorylation *in vivo* is consistent with the TGF-β-dependent activation of TβRI by TβRII. The mechanism of ShcA phosphorylation by TGF-β receptors thus recapitulates that of Smad activation. However, Smad proteins are phosphorylated only on serine (Macias-Silva *et al*, 1996).

Unlike TβRII (Lawler *et al*, 1997), neither tyrosine kinase activity nor tyrosine phosphorylation have been reported for

Figure 8 Erk activation by TGF-β is inhibited by inactive ShcA mutants and ShcA downregulation. 3T3-Swiss cells were infected with adenoviruses expressing GFP (control), HA-tagged $p52^{ShcA}$ lacking the PTB domain ($p52^{ShcA}\Delta PTB$), or HA-tagged $p52^{ShcA}$ lacking the SH2 domain ($p52^{ShcA}\Delta SH2$). Cells were then stimulated with TGF-β for the indicated times and lysed. (**A**) TGF-β-induced tyrosine phosphorylation of endogenous ShcA is attenuated by expression of $p52^{ShcA}$ truncations. Endogenous ShcA was immunoprecipitated and subjected to phosphotyrosine Western analysis (above). HA immunoblotting of this membrane confirmed expression of $p52^{ShcA}$ truncations and equivalent precipitation efficiency (below). (**B**) TGF-β-induced Grb2 association with endogenous ShcA is attenuated by $p52^{ShcA}$ truncations. The membrane in (A) was subjected to Grb2 Western analysis. (**C**) TGF-β-induced Raf phosphorylation is attenuated by $p52^{ShcA}$ truncations. Cell lysates from the experiment shown in (A) were subjected to phosphoRaf Western blot. (**D**) TGF-β-induced Erk phosphorylation is attenuated by $p52^{ShcA}$ truncations. The membrane shown in (C) was subjected to phosphoErk1/2 Western blot (above). The membrane was reprobed for GAPDH to confirm equivalent loading (below). (**E**) TGF-β-induced Erk activation is attenuated by ShcA silencing and restored by ectopic expression of wild-type, but not mutant $p52^{ShcA}$. 3T3-Swiss cells were exposed to an siRNA targeted to the common region of ShcA together with adenoviruses encoding either green fluorescent protein (GFP), wild-type $p52^{ShcA}$, or $p52^{ShcA}$ in which the Grb2 binding sites are mutated. Both $p52^{ShcA}$ constructs incorporate a silent mutation in the siRNA target site. PhosphoErk immunoblot of cells stimulated with TGF-β for the indicated times (above). ShcA immunoblot confirming partial downregulation and virus-mediated expression (below).

TβRI. However, the TβRII and TβRI kinases share structural similarities with tyrosine kinases (Manning *et al*, 2002). Moreover, two critical autophosphorylated tyrosines in TβRII (Tyr^{336} in subdomain V and Tyr^{424} in subdomain VIII) are conserved in TβRI. TβRII, like most dual specificity kinases (Hanks and Hunter, 1995), has low levels of tyrosine relative to serine-threonine kinase activity (Lawler *et al*, 1997), and this is also true for TβRI. The tyrosine kinase activities of dual specificity kinases also tend to manifest as autophosphorylation (Hanks and Hunter, 1995), as observed for TβRII (Lawler *et al*, 1997). This may also be true for TβRI, although it might also be phosphorylated by TβRII. In either case, TβRI tyrosine phosphorylation may enable its association with the ShcA PTB domain (Kavanaugh and Williams, 1994). Whereas the canonical PTB domain-binding NPXpY motif is not present in TβRI, a structurally similar motif is recognised and conserved in both TβRI and TβRII. Motifs that associate with the ShcA PTB domain typically contain a large hydrophobic residue, a phosphotyrosine located five amino acids downstream, and intervening arginine and proline residues that impose a β-turn (Trub *et al*, 1995). In TβRII, this structure is provided by the autophosphorylated Tyr^{424} (Lawler *et al*, 1997) located five bases downstream of a Val. Tyr^{424} and Val^{419} are highly conserved across TβRI and TβRII and correspond to Tyr^{378} and Val^{373} in human TβRI (Huse *et al*, 1999). Among the dual specificity kinases, only MEK kinases are known to phosphorylate substrates on tyrosine and serine (Lindberg *et al*, 1992), as we now report for TβRI.

Receptor tyrosine kinases initiate ShcA signalling by phosphorylating $p52^{ShcA}$ at tyrosines 239, 240, and 317 (van der Geer *et al*, 1996), thereby creating binding sites for Grb2. The resulting ShcA/Grb2/Sos complex then translocates to the plasma membrane and activates Ras (Lotti *et al*, 1996). Similarly, TGF-β induces low levels of ShcA/Grb2 association that correlate with low levels of Erk activation, suggesting that the same tyrosines mediate TGF-β signalling. This inference is supported by our finding that siRNA-mediated ShcA downregulation attenuated TGF-β-induced Erk activation, and that this attenuation was reversed by ectopic expression of wild-type $p52^{ShcA}$, but not $Tyr^{239,240,317}$Phe $p52^{ShcA}$. Thus, Erk activation by TGF-β stimulation is at least partially mediated by ShcA phosphorylation at these tyrosines, with consequent ShcA/Grb2/Sos complex formation. Furthermore, TGF-β-induced Erk activation was inhibited by ShcA truncations that may sequester wild-type ShcA. However, mutation of $p52^{ShcA}$ at all three tyrosines does not fully abrogate EGF-induced Erk activation (Thomas and Bradshaw, 1997); in fact, enhanced MAP kinase activity suggests alternative Erk activation and compensatory regulation. Such mechanisms may explain the increased Raf phosphorylation in cells expressing $p52^{ShcA}$ΔPTB and the increased basal Erk2 phosphorylation in cells expressing $p52^{ShcA}$ΔSH2 (Figure 8C and D). Conversely, ShcA signalling that is dependent on $Tyr^{239,240}$ phosphorylation, but independent of Ras activation has been reported (Gotoh *et al*, 1996). These alternative mechanisms may contribute to Erk activation by TβRI.

It is unclear how serine phosphorylation affects ShcA function. Because TGF-β can attenuate Erk activation by receptor tyrosine kinases (Berrou *et al*, 1996), we speculate that ShcA serine phosphorylation may inhibit its participation in tyrosine kinase signalling. TGF-β-induced serine phosphorylation may also activate ShcA functions that do not lead to MAP kinase activation. Upon serine phosphorylation of its CH2 domain, $p66^{ShcA}$ mediates an oxidative stress response. Cells deficient in $p66^{ShcA}$ are resistant to H_2O_2-induced cell death, and susceptibility is restored by expressing wild-type $p66^{ShcA}$, but not a serine phosphorylation-deficient mutant (Migliaccio *et al*, 1999). As TGF-β enhances H_2O_2-induced apoptosis (Yasuda *et al*, 2003) and is expressed in response to H_2O_2 (Frippiat *et al*, 2001), we speculate that TGF-β-induced ShcA phosphorylation may contribute to the oxidative stress response.

TGF-β signalling may also depend on the relative expression of ShcA isoforms. $p52^{ShcA}$ mediates Ras activation by receptor tyrosine kinases (Pelicci *et al*, 1992), whereas $p66^{ShcA}$ antagonises Ras and Erk activation (Migliaccio *et al*, 1997). Moreover, $p66^{ShcA}$ expression varies widely during fetal development (Lee *et al*, 1998). As TGF-β receptors phosphorylate all three ShcA isoforms, their activation of Erk MAP kinases may be regulated by ShcA isoform expression.

The repertoire of TGF-β signalling should now be expanded to include responses primarily associated with receptor tyrosine kinases. The identification of ShcA activation as a direct link between TGF-β stimulation and Erk MAP kinase signalling further confirms the highly interconnected nature of intracellular signalling.

Materials and methods

Cell culture, transfections, and adenoviral infections

3T3-Swiss, HEK-293, COS, and Mv1Lu cells (ATCC, Manassas, VA) were cultured in MEM-α or DMEM with 10% fetal calf serum. Cells were grown to 75% confluence in six-well plates before transfection or infection. Transfections were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). For adenoviral infections, 5×10^7 p.f.u. were added to each well (MOI = 20). Cells were rested for 48 h, starved overnight, and stimulated with 4 ng/ml TGF-β1 (Leinco, St Louis, MO) or 20 ng/ml EGF (R&D Systems, Minneapolis, MN). In selected experiments, cells were treated with 10 μM SB431542 (Sigma, St Louis, MO). To downregulate ShcA expression, cells cultured to 60% confluence in six-well plates were treated for 4 h with 3 μl/well of 50 μM siRNA suspended in 4 μl Oligofectamine (Invitrogen). Cells were incubated overnight with adenoviruses encoding siRNA-resistant $p52^{ShcA}$ or GFP, then rested 1 day before starvation and TGF-β stimulation.

Mammalian expression plasmids and siRNA-mediated ShcA downregulation

$p66^{ShcA}$ and $p52^{ShcA}$ cDNAs were isolated from a fetal mouse λgt-11 library (Clontech, Palo Alto, CA) using ShcA antibody. High-fidelity PCR (Advantage HF, Clontech or pFU, Stratagene, La Jolla, CA) was used to truncate proteins, add restriction sites, append transcription initiation or termination sites, and incorporate epitope tags. ShcA deletion mutants were generated by truncating the coding region along domain boundaries (Luzi *et al*, 2000).The $p52^{ShcA}$ΔSH2 mutant encodes amino acids 1–373 with a C-terminal HA-tag and stop codon. The $p52^{ShcA}$ΔPTB truncation encodes amino acids 208–469 with a start codon, Kozak sequence and C-terminal HA-tag. Vectors expressing the PTB (amino acids 1-233) or CH1 (amino acids 234–373) domains incorporate start codons, Kozak sequences, C-terminal HA tags, and stop codons. For mammalian expression, constructs were subcloned into pRK5 (BD Pharmingen, San Diego, CA) at *Eco*RI and *Hin*dIII.

Constructs expressing TβRI and TβRII, their cytoplasmic domains (TβRIc and TβRIIc), and the cytoplasmically truncated TβRII (DN-TβRII) have been described (Feng *et al*, 1995). The TβRIc KR kinase was inactivated by mutating Lys^{230} to Arg (Chen *et al*, 1995). Similarly, TβRIIc KR was inactivated by mutating Lys^{277} to Arg (Wrana *et al*, 1992). C-terminal Myc- or FLAG-tags were added before subcloning into pRK5 at *Eco*RI and *Hin*dIII. Plasmids were purified by endotoxin-free maxiprep (Qiagen, Chatsworth, CA).

Dominant inhibitory ShcA adenoviruses were constructed by subcloning HA-tagged $p52^{ShcA}\Delta SH2$ and $p52^{ShcA}\Delta PTB$ into pShuttle-CMV-GFP (Stratagene) at *Kpn*I and *Hin*dIII. Constructs incorporating myc epitopes and either TβRIc or TβRIIc were subcloned into pShuttle-CMV-GFP at *Hin*dIII and *Not*I. A control virus expressing only GFP was also produced. The plasmids were recombined with pAdEasy-1 (Stratagene) in *E. coli* BJ5183 and expanded in HEK 293 packaging cells.

ShcA expression was silenced with siRNA against nucleotides 677–697 of $p52^{ShcA}$, a region common to all three isoforms (Kisielow *et al*, 2002). To restore $p52^{ShcA}$ function, site-directed mutagenesis (QuikChange, Stratagene) was used to generate a wild-type $p52^{ShcA}$ transcript with the siRNA target mutated from CTGTCA to CTCAGT, thereby leaving the amino-acid sequence unchanged. A second $p52^{ShcA}$ construct was developed in which the Grb2 docking sites at tyrosines 239, 240, and 317 were mutated to phenylalanines. Constructs were subcloned into pShuttle as above.

Protein expression in E. coli and insect cells

For bacterial expression of $p66^{ShcA}$, $p52^{ShcA}$, and the cytoplasmic domains such as TβRIc, TβRIc KR, TβRIIc, and TβRIIc KR, the coding sequences were flanked with *Eco*RI and *Not*I sites using high-fidelity PCR and cloned into the pGEX-6P-1 GST fusion vector (Pharmacia Amersham, Piscataway, NJ). The plasmids were transformed into *E. coli* BL21 De3 Lys *S* (Stratagene), the expressed fusion proteins adsorbed to glutathione Sepharose 4B, and the GST segment removed with Factor Xa protease (Pharmacia Amersham).

A constitutively active chimera of the TβRI and TβRII cytoplasmic domains (Feng and Derynck, 1996) was produced in Sf9 cells using the Bac-To-Bac baculovirus system (Invitrogen). The sequence encoding the RII-RI chimera was cloned from pRK5-(RII-I)C into pFastBac I (Invitrogen) together with a C-terminal myc epitope. Chimeras incorporating inactivating point mutations in one or both kinase domains were made using PCR-based mutagenesis. In the wtRII-RIKR chimera, Lys^{230} in the TβRI kinase domain was replaced by Arg (Chen *et al*, 1995). In RIIKR-wtRI, Lys^{277} in the TβRII kinase domain was replaced by Arg (Wrana *et al*, 1992). The RIIKR-RIKR chimera incorporated both mutations. Chimeras were purified using myc (9E10) affinity matrix (Covance Babco, Berkeley, CA).

Immunoprecipitations and immunoblotting

Anti-phosphotyrosine, anti-ShcA, anti-Sos, anti-Grb1, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies were from BD Transduction Laboratories (Lexington, KY). Anti-phosphoErk1/2 antibody was from Biosource (Camarillo, CA). Anti-myc, anti-HA, and anti-FLAG antibodies were provided by Covance Babco. Anti-TβRI and anti-phosphoRaf antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-TβRII antibody was from Upstate (Charlottesville, VA). Immobilised anti-mouse IgG and anti-rabbit IgG antibodies were from EY Laboratories (San Mateo, CA).

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM $NaVO_4$, 10 mM NaF and Complete protease inhibitor (Roche, Indianapolis, IN)), and the lysate protein concentrations quantified and equalised using Micro BCA (Pierce, Rockford, IL). For phosphotyrosine and co-precipitation analyses, proteins were precipitated overnight at 4°C with 1 μg primary antibody and 10 μl immobilised anti-mouse or anti-rabbit IgG (EY Laboratories) per 100 μg of total protein. Immunoblots were visualised by chemiluminescence (Pierce).

ShcA kinase assays

ShcA phosphorylation by TGF-β receptors *in vitro* was evaluated using myc-tagged TβRI or TβRII immunoprecipitated from transfected 293 cells, GST-fused TβRIc or TβRIIc generated in *E. coli*, or chimeric TβRII-TβRI cytoplasmic domains. Receptor proteins were incubated with $p52^{ShcA}$ or $p66^{ShcA}$ purified from *E. coli* (see above) in 25 mM HEPES (pH 7.4), 2 mM $MnCl_2$, 10 mM $MgCl_2$, 0.01% Triton X-100, 100 μM Na_3VO_4, 50 mM NaF, 20 μM DTT, 10 μM ATP, and 5 μCi/reaction γ-^{32}P ATP at 37°C for 30 min. Labelled reaction products were visualised by autoradiography.

Phosphoamino acid analyses

Radiolabelled proteins were resolved by SDS–PAGE, transferred to polyvinylidene difluoride membrane and isolated by autoradiography and band excision. Proteins were hydrolysed in 6 N HCl at 110°C for 1 h, resolved by two-dimensional thin-layer electrophoresis (Cooper *et al*, 1983), and visualised by autoradiography.

Interaction of ShcA with cell surface TGF-β receptors

ShcA association with cell surface TGF-β receptors was assessed as described previously (Lyons *et al*, 1991). Briefly, 3T3-Swiss cells were incubated with ^{125}I-TGF-β1 for 10 min on ice and treated with 100 μM disuccimidyl suberate (Pierce) for 15 min. Cells were solubilised in 1% Triton X-100, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA before ShcA immunoprecipitation. Labelled receptors were visualised by autoradiography.

Signalling assays

Association of ShcA with Grb2 or Sos was assessed by ShcA immunoprecipitation followed by Grb2 or Sos immunoblotting. Raf phosphorylation was evaluated by phosphoRaf immunoblotting. Erk1/2 phosphorylation was assayed by phosphoErk1/2 immunoblot. Erk1/2 activity was assessed by *in vitro* kinase assay (Cano *et al*, 1995) using $Elk1_{307-428}$ fused to GST as substrate (provided by RA Hipskind). Cells were lysed in 10 mM Tris (pH 7.05), 30 mM $Na_4P_2O_7$, 1% Triton X-100, 50 mM NaCl, 5 mM $ZnCl_2$, 100 μM Na_3VO_4, 50 mM NaF, 1 mM DTT, 5 μM okadaic acid and Complete protease inhibitor. Lysates were equalised for protein content and Erk MAP kinases precipitated with Elk1-GST coupled to glutathione-agarose at 4°C for 4 h. Reactions were performed in 20 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 20 mM β-glycerophosphate, 10–20 mM *p*-nitrophenylphosphate, 100 μM Na_3VO_4, 50 nM okadaic acid, 50 mM NaF, 2 mM DTT, Complete protease inhibitor, 20 mM ATP, and 5 μCi/reaction γ-^{32}P ATP at 25°C for 30 min. Labelled complexes were visualised by autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Attisano L, Wrana JL (2002) Signal transduction by the TGF-β superfamily. *Science* **296:** 1646–1647

Berrou E, Fontenay-Roupie M, Quarck R, McKenzie FR, Lévy-Toldano S, Tobelem G, Bryckaert M (1996) Transforming growth factor β1 inhibits mitogen-activated protein kinase induced by basic fibroblast growth factor in smooth muscle cells. *Biochem J* **316:** 167–173

Cano E, Hazzalin CA, Kardalinou E, Buckle RS, Mahadevan LC (1995) Neither ERK nor JNK/SAPK MAP kinase subtypes are essential for histone H3/HMG-14 phosphorylation or c-fos and c-jun induction. *J Cell Sci* **108:** 3599–3609

Chen RH, Moses HL, Maruoka EM, Derynck R, Kawabata M (1995) Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II transforming growth factor-β receptors. *J Biol Chem* **270:** 12235–12241

Cooper JA, Sefton BM, Hunter T (1983) Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol* **99:** 387–402

Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I (2005) Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-β1 involves MAPK, Smad and AP-1 signalling pathways. *J Cell Biochem* **95:** 918–931

Derynck R, Akhurst RJ, Balmain A (2001) TGF-β signaling in tumor suppression and cancer progression. *Nat Genet* **29:** 117–129

Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425:** 577–584

Feng XH, Derynck R (1996) Ligand-independent activation of transforming growth factor (TGF) β signaling pathways by heteromeric cytoplasmic domains of TGF-β receptors. *J Biol Chem* **271:** 13123–13129

Feng XH, Filvaroff EH, Derynck R (1995) Transforming growth factor-β (TGF-β)-induced down-regulation of cyclin A expression requires a functional TGF-β receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J Biol Chem* **270:** 24237–24245

Frippiat C, Chen QM, Zdanov S, Magalhäes JP, Remacle J, Toussaint O (2001) Subcytotoxic H_2O_2 stress triggers a release of transforming growth factor-β1, which induces biomarkers of cellular senescence of human diploid fibroblasts. *J Biol Chem* **276:** 2531–2537

Gotoh N, Muroya K, Hattori S, Nakamura S, Chida K, Shibuya M (1995) The SH2 domain of Shc suppresses EGF-induced mitogenesis in a dominant negative manner. *Oncogene* **11:** 2525–2533

Gotoh N, Tojo A, Shibuya M (1996) A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3. *EMBO J* **15:** 6197–6204

Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9:** 576–596

Huse M, Chen YG, Massagué J, Kuriyan J (1999) Crystal structure of the cytoplasmic domain of the type I TGFβ receptor in complex with FKBP12. *Cell* **96:** 425–436

Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **62:** 65–74

Kavanaugh WM, Williams LT (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* **266:** 1862–1865

Kisielow M, Kleiner S, Nagasawa M, Faisal A, Nagamine Y (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J* **363:** 1–5

Kretzschmar M, Doody J, Timokhina I, Massagué J (1999) A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. *Genes Dev* **13:** 804–816

Lai KM, Pawson T (2000) The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Genes Dev* **14:** 1132–1145

Lawler S, Feng XH, Chen RH, Maruoka EM, Turck CW, Griswold-Prenner I, Derynck R (1997) The type II transforming growth factor-β receptor autophosphorylates not only on serine and threonine but also on tyrosine residues. *J Biol Chem* **272:** 14850–14859

Lee MK, Zhao J, Smith S, Tefft JD, Bringas Jr P, Hwang C, Warburton D (1998) The Shc 66 and 46 kDa isoforms are differentially downregulated at parturition in the fetal mouse lung. *Pediatr Res* **44:** 850–859

Lindberg RA, Quinn AM, Hunter T (1992) Dual-specificity protein kinases: will any hydroxyl do? *Trends Biochem Sci* **17:** 114–119

Lotti LV, Lanfrancone L, Migliaccio E, Zompetta C, Pelicci G, Salcini AE, Falini B, Pelicci PG, Torrisi MR (1996) Shc proteins are localized on endoplasmic reticulum membranes and are redistributed after tyrosine kinase receptor activation. *Mol Cell Biol* **16:** 1946–1954

Luzi L, Confalonieri S, di Fiore PP, Pelicci PG (2000) Evolution of Shc functions from nematode to human. *Curr Opin Genet Dev* **10:** 668–674

Lyons RM, Miller DA, Graycar JL, Moses HL, Derynck R (1991) Differential binding of transforming growth factor-β1, -β2, and -β3 by fibroblasts and epithelial cells measured by affinity cross-linking of cell surface receptors. *Mol Endocrinol* **5:** 1887–1896

Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL (1996) MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87:** 1215–1224

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* **298:** 1912–1934

Massagué J (2000) How cells read TGF-β signals. *Nat Rev Mol Cell Biol* **1:** 169–178

Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG (1999) The $p66^{Shc}$ adaptor protein controls oxidative stress response and life span in mammals. *Nature* **402:** 309–313

Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti Furga G, Pawson T, di Fiore PP, Lanfrancone L, Pelicci PG (1997) Opposite effects of the $p52^{Shc}$/$p46^{Shc}$ and $p66^{Shc}$ splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. *EMBO J* **16:** 706–716

Mucsi I, Skorecki KL, Goldberg HJ (1996) Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-β1 on gene expression. *J Biol Chem* **271:** 16567–16572

Mulder KM (2000) Role of Ras and Mapks in TGFβ signaling. *Cytokine Growth Factor Rev* **11:** 23–35

Olsson N, Piek E, Sundstrom M, ten Dijke P, Nilsson G (2001) Transforming growth factor-β-mediated mast cell migration depends on mitogen-activated protein kinase activity. *Cell Signal* **13:** 483–490

Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Pawson T, Pelicci PG (1992) A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70:** 93–104

Qi M, Elion EA (2005) MAP kinase pathways. *J Cell Sci* **118:** 3569–3572

Ravichandran KS (2001) Signaling via Shc family adapter proteins. *Oncogene* **20:** 6322–6330

Simeone DM, Zhang L, Graziano K, Nicke B, Pham T, Schaefer C, Logsdon CD (2001) Smad4 mediates activation of mitogen-activated protein kinases by TGF-β in pancreatic acinar cells. *Am J Physiol Cell Physiol* **281:** C311–C319

Thomas D, Bradshaw RA (1997) Differential utilization of ShcA tyrosine residues and functional domains in the transduction of epidermal growth factor-induced mitogen-activated protein kinase activation in 293T cells and nerve growth factor-induced neurite outgrowth in PC12 cells. Identification of a new Grb2-2-Sos1 binding site. *J Biol Chem* **272:** 22293–22299

Trub T, Choi WE, Wolf G, Ottinger E, Chen Y, Weiss M, Shoelson SE (1995) Specificity of the PTB domain of Shc for β turn-forming pentapeptide motifs amino-terminal to phosphotyrosine. *J Biol Chem* **270:** 18205–18208

van der Geer P, Wiley S, Gish GD, Pawson T (1996) The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein-protein interactions. *Curr Biol* **6:** 1435–1444

Ventura A, Maccarana M, Raker VA, Pelicci PG (2004) A cryptic targeting signal induces isoform-specific localization of $p46^{Shc}$ to mitochondria. *J Biol Chem* **279:** 2299–2306

Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massagué J (1992) TGFβ signals through a heteromeric protein kinase receptor complex. *Cell* **71:** 1003–1014

Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH (1994) Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. *J Biol Chem* **269:** 20172–20178

Yasuda K, Aoshiba K, Nagai A (2003) Transforming growth factor-β promotes fibroblast apoptosis induced by H_2O_2. *Exp Lung Res* **29:** 123–134