Bifurcation of Cell Migratory and Proliferative Signaling by the Adaptor Protein Shc

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Abstract. Cytokines and extracellular matrix proteins initiate signaling cascades that regulate cell migration and proliferation. Evidence is provided that the adaptor protein Shc can differentially regulate these processes. Specifically, under growth factor–limiting conditions, Shc stimulates haptotactic cell migration without affecting anchorage-dependent proliferation. However, when growth factors are present, Shc no longer influences cell migration; rather, Shc is crucial for DNA synthesis. Mutational analysis of Shc demonstrates that, while tyrosine phosphorylation is required for both DNA synthesis and cell migration, the switch in Shc signaling is associated with differential use of Shc's phosphotyrosine interacting domains; the PTB domain regulates haptotaxis, while the SH2 domain is selectively required for proliferation.

Key words: Shc • cell migration • mitogenesis • SH2 • PTB

Cell migration and proliferation are essential to angiogenesis, embryonic development and wound healing. It has become clear that cell migration and proliferation depend on intracellular signals propagated by growth factors and adhesion proteins within the extracellular matrix (ECM)[1]. However, cell migration and DNA synthesis do not necessarily occur simultaneously. For example, neural crest cells migrate over long distances in the embryo yet fail to proliferate (Perris, 1997). During wound repair, keratinocytes migrate into the wound before entry into the cell cycle (Martin, 1997). Recent studies have determined that cell migration and proliferation utilize many of the same intracellular signaling pathways such as activation of Rho family proteins and the extracellular signal-related matrix (ERK) cascade (Pages et al., 1993; Olson et al., 1995; Anand-Apte et al., 1997; Klemke et al., 1997). However, signaling molecules likely exist that allow cells to differentially regulate cell migration and proliferation. Evidence is provided in this report that the adaptor protein Shc serves such a purpose.

The Shc family of adaptor proteins consists of multiple protein-protein interaction domains: an amino-terminal phosphotyrosine binding (PTB) domain a central collagen homology (CH) domain and a carboxy-terminal Src homology 2 (SH2) domain (Pelicci et al., 1992; Blaikie et al., 1994). Shc exists in three isoforms of 46, 52, and 66 kD, and was found to associate with and became tyrosine phosphorylated by the EGF receptor and to be capable of inducing anchorage-independent growth (Pelicci et al., 1992). After growth factor stimulation, p52 Shc is recruited to activated tyrosine kinase receptors through either its PTB or its SH2 domain, which leads to phosphorylation at tyrosine residues 239, 240, and 317 within the CH domain (Rozakis-Adcock et al., 1992; Gotoh et al., 1996; van der Geer et al., 1996). Tyrosine-phosphorylated Shc thus is able to recruit Grb2/SOS through a binding event between the Grb2 SH2 domain and Shc phosphotyrosine residues (Pelicci et al., 1992; Rozakis-Adcock et al., 1992), ultimately resulting in activation of Ras, the ERK cascade, and mitogenesis (Bonfini et al., 1996). Recent reports demonstrate that Shc also potentiates integrin signaling. For example, integrin ligation results in activation of tyrosine kinases such as Src, Fyn, and focal adhesion kinase (FAK) that phosphorylate Shc leading to Ras activation and entry into the cell cycle (McGlade et al., 1992; Mainiero et al., 1995; Wary et al., 1996, 1998; Schlaepfer et al., 1998).

In addition to their role in regulating DNA synthesis, growth factor receptors and integrins play a crucial role in cell migration and invasion (Klemke et al., 1994; Huttenlocher et al., 1995). This process involves rearrangement of

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[1] *Abbreviations used in this paper:* ANOVA, analysis of variance; BrdU, bromodeoxyuridine; CH, collagen homology; ECM, extracellular matrix; ERK, extracellular signal-related kinase; FAK, focal adhesion kinase; SH2, Src homology 2; PTB, phosphotyrosine binding.

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the actin cytoskeleton, the formation of new integrin substratum contacts, cell contraction, and release of preexisting cell–matrix contacts at the trailing edge (Lauffenburger and Horwitz, 1996). Previous reports have suggested a role for Shc in this process (Pelicci et al., 1995; Nolan et al., 1997). Therefore, experiments were designed to evaluate the role of Shc in cell migration and DNA synthesis. In this report, evidence is presented that Shc plays a critical role in regulating these cell biological events. Shc is required for cell migration, but not proliferation, when growth factors are limiting. However, in the presence of growth factors Shc no longer influences cell migration, but rather, is essential for DNA synthesis. A mutational analysis of Shc has helped to elucidate how a given adaptor protein can differentially activate cell migration and proliferation.

Materials and Methods

Antibodies and Reagents

Rabbit polyclonal antibodies to Shc were purchased from Transduction Laboratories. Mouse monoclonal Shc antibodies were from Santa Cruz Biotechnology. Anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology. Shc cDNAs subcloned into pCDNA3.1HisC were previously described (Ricketts et al., 1999). TRITC-conjugated phalloidin was obtained from Sigma. 6F1 was a gift of Dr. Virgil Woods (UCSD, La Jolla, CA) and mAb 1973Z was obtained from Chemicon.

Cell Culture

FG-M cells were maintained in RPMI supplemented with 10% FBS, 1 μg/ml gentamicin, and 0.4 mM glutamine (gentamycin/glutamine; Sigma). Cells were used from passage 8 to passage 13. Cos-7 cells were maintained in DME supplemented with 10% FBS, 0.4 mM glutamine, and 1 μg/ml gentamicin. Cells were used from passage 6 to passage 20 and were mycoplasma free during the course of these studies.

Transfection

Cells were transfected with *lac z* (to identify transfected cells) and the indicated constructs on 10-cm tissue culture plates using LipofectAMINE (GIBCO BRL) per the manufacturer's instructions. Cos-7 cells were incubated in the transfection mixture for 5 to 7 h and FG-M cells were allowed to incubate for 15 h. At the end of this time, cells were returned to full growth medium for either 15 h (Cos-7 cells) or 8 h (FG-M) cells, and serum deprived for 20–24 h before use in experiments.

Generation of p52 Shc Stable Cell Line

Cos-7 cells were transfected with wild-type, murine p52 Shc cDNA subcloned into pCDNA3.1HisC using LipofectAMINE per the manufacturer's instructions. 3 d later, cells were passaged into growth medium supplemented with 500 μg/ml G418 to select for p52 Shc-expressing cells. After selection, cells were seeded at low density into 96-well plates, single colonies were isolated with a cloning ring, and expanded for two passages before experimental use.

Immunofluorescence

Acid-washed coverslips were coated with collagen (10 μg/ml) and nonspecific binding was blocked with 0.5% RIA grade BSA (Sigma). Serum-deprived cells were harvested as described below and seeded onto collagen-coated coverslips. Cells were allowed to spread for 2 h, then were fixed in 3.7% formaldehyde in PBS, permeabilized for 1 min in 0.01% Triton X-100, and actin was visualized by incubating the coverslips for 1 h with 2 μg/ml rhodamine conjugated phalloidin (Sigma). Coverslips were washed three times in PBS and three times in deionized water then mounted with gelvatol. Images were acquired with Bio-Rad MRC1024 confocal system using Bio-Rad Lasersharp software and a 63× objective on a Zeiss Axiovert 100. Images were processed with Adobe Photoshop.

Migration Assays

Motility experiments were performed as previously described (Klemke et al., 1998). In brief, Boyden chambers (Millipore Millicells, 8-mm pore size) were coated on either their lower surfaces with 10 μg/ml of the indicated extracellular matrix proteins for 2 h at 37°C. Chambers were then placed in 24-well culture dishes containing either serum-free DME supplemented with gentamicin, glutamine, and 0.5% BSA (RIA grade, "migration buffer"; Sigma) or migration buffer supplemented with 100 ng/ml EGF (mouse receptor grade; UBI) or 25 mg/ml insulin (human recombinant insulin; Boehringer Mannheim Biochemica) as indicated. Cells were harvested in buffer consisting of Hanks' balanced salt solution, 25 mM Hepes, 5 mM EDTA, and 0.01% trypsin. Next, cells were washed twice in serum-free DME containing 0.5% BSA and 150,000 cells were loaded into the upper portion of the Boyden chambers. Migrations were allowed to proceed for 4–6 h. At the end of this time, nonmigratory cells were removed with a cotton swab, and chambers were stained for β-galactosidase activity (to identify transfected cells) or stained with crystal violet. Migration was quantitated by blind counting the number of migratory cells on the lower surface of the membrane of at least two fields per chamber using a 20× objective. Statistical analysis was performed using InStat (Graph Pad) by pooling the data from multiple independent experiments.

Modified Migration Assays

Integrin antibody blocking experiments were performed as described above except that Cos-7 cells were mixed with the indicated anti-integrin antibodies immediately before loading into Boyden chambers; experiments were then allowed to proceed as above for 4 h. Random migration experiments were performed using chambers that had been coated with extracellular matrix protein on either their lower surfaces (haptotaxis) or their upper and lower surfaces (random migration). Video time-lapse microscopy was also attempted to assess random migration in serum-deprived cells on extracellular matrix protein; however, during the course of the experiment (6 h), Cos-7 cells did not migrate using this method.

Replating Assays, Immunoprecipitation, and Western Blotting

Petri dishes were coated with extracellular matrix proteins as indicated. Nonspecific binding of cells to plates were blocked by incubating with heat-denatured BSA for 30 min. Next, serum-deprived cells were harvested as described above and either left in suspension for 20 min or plated onto matrix-coated, blocked plates for the indicated times and harvested in a lysis buffer containing 10 mM Tris, pH 7.6, 150 mM sodium chloride, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, and 5 mM EDTA. The following inhibitors were added immediately before use: complete protease inhibitor (1 tablet/20 ml; Boehringer Mannheim), 1 mM PMSF, 2.5 mM sodium orthovanadate. Shc was either immunoprecipitated using a polyclonal Shc antibody (Transduction Labs) or His-tagged Shc was isolated using nickel agarose beads (Ni-NTA agarose; Qiagen). Phosphotyrosine was detected using the monoclonal anti-phosphotyrosine antibody, 4G10. Shc was detected using a either a monoclonal or a polyclonal Shc antibody. Bands were visualized by chemiluminescence (Super Signal; Pierce). Expression levels of Shc mutants in migration assays was determined by reserving an aliquot of cell lysate from the migration assay and analyzing it by Western blot with a polyclonal Shc antibody.

DNA Synthesis Assay

Cells were transfected as described above on coverslips. 24 h later, cells were serum starved overnight and stimulated with either serum-free DME or DME containing 100 ng/ml EGF. 12 h later, cells were incubated for an additional 6 h with bromodeoxyuridine (BrdU) to identify newly synthesized DNA. Coverslips were then washed with PBS and fixed in 3.7% formaldehyde. BrdU was quantitated in transfected cells by staining with a rat anti-BrdU antibody (Amersham) and a mouse antiExpress antibody (Invitrogen, to identify proteins expressed from pCDNA3.1HisC).

Results

A Stable p52 Shc Expressing Cell Line Has Decreased Stress Fibers and Increased Migratory Capacity

Recent reports have identified a role for p52 Shc in inte-

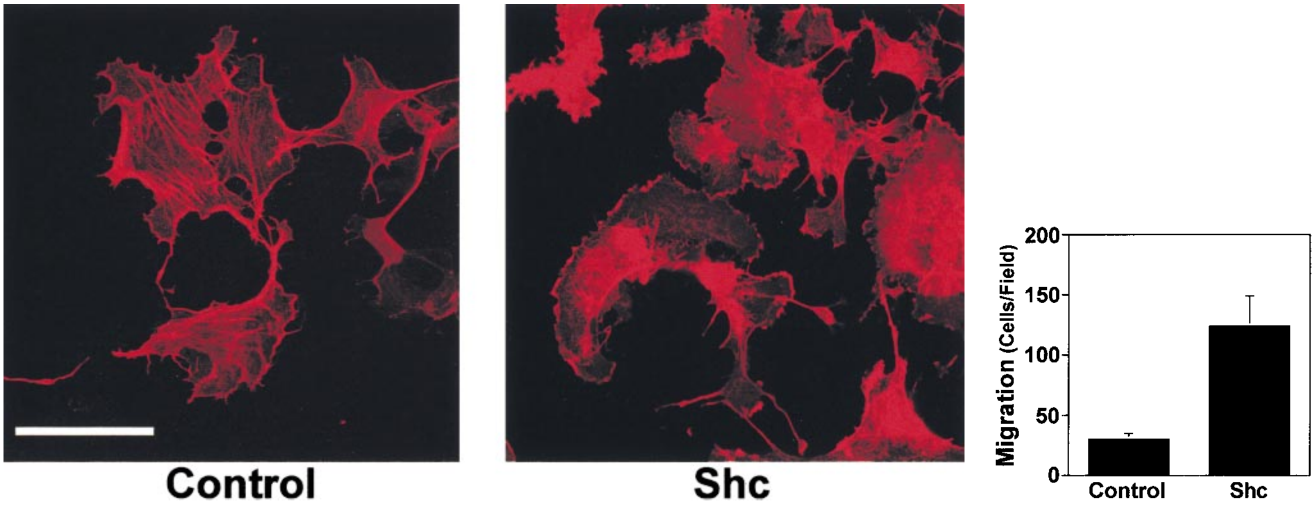

Figure 1. Decreased actin organization and increased migration of Shc expressing Cos-7 cells. (Left) Serum-deprived control and Shc stably expressing cells were harvested, plated on collagen-coated coverslips for 2 h, fixed, permeabilized, and stained with rhodamine phalloidin. The images shown are single confocal optical sections taken with a 63× objective (left). The scale bar is equal to 50 μm. (Right) Migration of parental vs Shc-expressing Cos-7 cells. Serum-deprived cells were allowed to migrate for 4 h in Boyden chambers towards a collagen matrix. Data from one of four independent experiments are shown (mean ± SE). The enhanced migration of the Shc cell line was found to be statistically significant ($P < 0.05$ by Student's *t* test). Random migration towards BSA-coated chambers was always $<2\%$ and subtracted from each value.

grin signaling leading to cell cycle progression (Wary et al., 1996). Since many of the signals that regulate mitogenesis also promote cell migration (Pages et al., 1993; Cowley et al., 1994; Olson et al., 1995; Anand-Apte et al., 1997; Klemke et al., 1997), we reasoned that Shc might also play a role in cell movement. To address this question, Cos-7 cells were stably transfected with wild-type murine p52 Shc cDNA. The resulting cells displayed an altered morphology from the parental cell line. Phalloidin staining of p52 Shc cells and parental Cos-7 cells revealed a decreased number of actin-containing stress fibers in Shc-expressing cells (Fig. 1), suggestive of a motile phenotype. The migratory capacity of the parent and stable p52 Shc cells was then assessed. Serum-deprived cells were allowed to migrate in the absence of added cytokines using Boyden chambers that had been coated on their lower surface with collagen. As shown in Fig. 1, p52 Shc stable cells were approximately three times more migratory than the parental cells. When the data from four independent experiments were analyzed this difference was found to be statistically significant ($P < 0.05$ by Student's *t* test), suggesting that Shc expression is associated with the haptotactic migratory phenotype of these cells.

To extend these findings, we assessed the motility of Cos-7 cells transiently expressing p52 Shc. Cells were serum-deprived and allowed to migrate on either collagen or vitronectin. As shown in Fig. 2, expression of p52 Shc was sufficient to promote cell migration to an extent similar to that seen with EGF, a strong migratory stimulus for these cells (Klemke et al., 1998). In addition, Shc promoted a similar migration response on collagen and vitronectin. Data from several experiments demonstrated that both of these responses were statistically significant ($P < 0.01$ by Student's *t* test). Cos-7 cells can use $\alpha2\beta1$ to migrate on collagen (Klemke et al., 1997) suggesting that Shc's ability to transmit integrin-mediated signals for migration may be more permissive than previously observed for cell cycle progression (Wary et al., 1996). In agreement with the motility results, ligation of integrins with either collagen or vitronectin resulted in tyrosine phosphorylation of wild-type p52 Shc indicating that both of these ECM proteins support Shc phosphorylation (Fig. 2).

Specific anti-integrin antibodies were used to determine the integrin dependence of Shc's effect upon motility. To determine the role of the collagen receptors $\alpha2\beta1$ and $\alpha1\beta1$ in this response, we first established concentrations of $\alpha1$ (mAb 1973Z) and $\alpha2\beta1$ (6F1) monoclonal antibodies that inhibited adhesion of Cos-7 cells to collagen (5 μg/ml of anti-$\alpha1$ and 10 μg/ml of anti-$\alpha2\beta1$, data not shown). Next, these concentrations of monoclonal antibodies were added to the cells immediately before placement in Boyden chambers. Both anti-$\alpha1$ antibodies and $\alpha2\beta1$ antibodies inhibited Shc's ability to promote cell migration; $\alpha2\beta1$ antibodies had a more pronounced effect (Fig. 3). These data establish that $\alpha2\beta1$ and Shc can signal along a common pathway resulting in cell movement. P4C10, an antibody directed against the shared $\beta1$ subunit, completely inhibited haptotaxis of mock and Shc transfected cells on collagen (data not shown). LM609, a function blocking monoclonal antibody to $\alpha v\beta3$, prevented Shc-stimulated migration towards vitronectin, but not on collagen (data not shown), further confirming that Shc requires integrins to effect cell movement.

To determine the nature of Shc-induced cell migration, cells were allowed to migrate in Boyden chambers under two experimental conditions. Chambers were either coated with collagen on their lower surface to enable cells to engage in haptotactic migration, or they were coated on both their upper and lower surfaces to evaluate the random motility of these cells. Shc was only able to signifi-

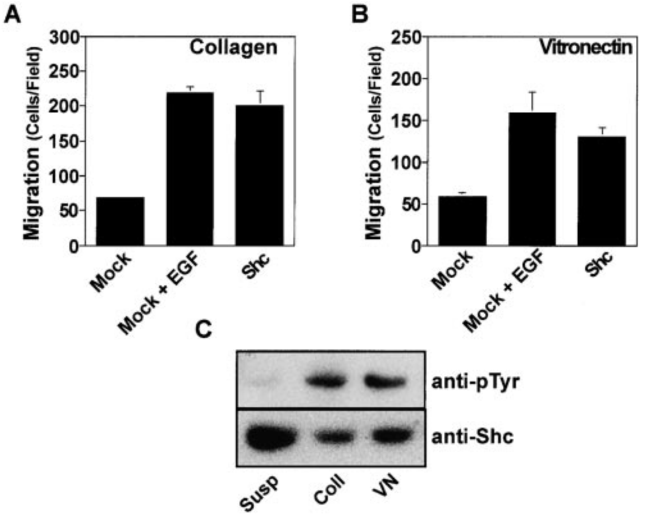

Figure 2. Shc stimulates migration on collagen and vitronectin. (Top) Cos-7 cells were either mock transfected (pcDNA3.1HisC plus *lac z)* or transfected with p52 Shc cDNA plus *lac z*. Cells were deprived of serum and migration was assessed as indicated with either migration media (mock, p52) or 100 ng/ml EGF (Mock+EGF) in the lower chamber. Migration was quantitated by enumerating the number of β-galactosidase positive cells/field using a 20× objective. The mean ± SE from a representative of five independent experiments is shown. (Bottom) Tyrosine phosphorylation of p52 Shc in response to integrin ligation. Serum-deprived cells expressing p52 Shc were harvested, then either held in suspension or replated on collagen or vitronectin for 1 h and lysed in RIPA buffer. His-tagged Shc was isolated with nickel agarose beads and analyzed for phosphotyrosine content by Western blot (mAb 4G10, upper autoradiogram). The blot was then stripped and reprobed with an anti-Shc antibody as described in Materials and Methods (lower autoradiogram).

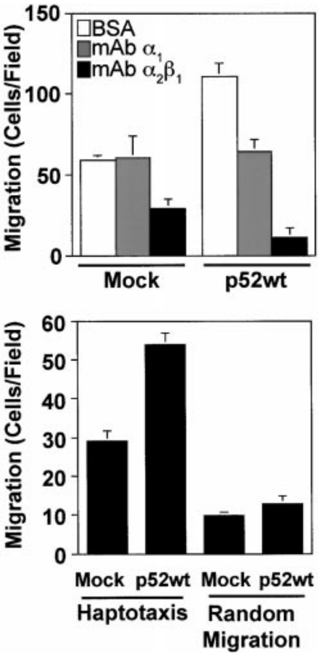

Figure 3. Shc stimulates haptotactic migration. (Top) Shc-stimulated cell migration is integrin dependent. Cells were processed for migration assay as described in Materials and Methods, except that cells were mixed with anti-integrin monoclonal antibodies as indicated before loading into Boyden chambers. Next, cells were allowed to migrate for 4 h and quantitated for migration as described above. Each bar represents the mean ± SE from one experiment representative of three with similar results. (Bottom) Serum-deprived mock or p52 Shc transfected Cos-7 cells were allowed to migrate on Boyden chambers coated on either their lower surface (haptotaxis) or both their upper and lower surfaces (random migration). Each bar represents the mean ± SE of a representative experiment performed in triplicate.

cantly enhance motility when chambers were coated on the lower surface, demonstrating that Shc promotes haptotaxis and not random migration (Fig. 3 b).

Structural Requirements for Shc-induced Cell Motility

Having established a role for Shc in haptotaxis, mutational analysis was performed to identify regions of Shc that mediate this response. Several point mutants were employed for these studies (Fig. 4). To address the role of Shc tyrosine phosphorylation in cell migration, increasing amounts of cDNA encoding mutant forms of Shc bearing tyrosine to phenylalanine point mutations at residues 239/240 (Shc Y239F/Y240F), residue 317 (Shc Y317F) or both residues 239/240 and residue 317 (ShcY239F/Y240F/Y317F, Shc 3YF) were expressed in Cos-7 cells. With the exception of Shc 3YF, these mutants were expressed to similar extents as shown in Fig. 5 A. Next, cells expressing these mutants were allowed to migrate on a collagen or vitronectin substrate. Only wt Shc was found to significantly enhance cell migration ($P < 0.01$ by analysis of variance [ANOVA] of six independent experiments). Neither Y239/240F nor Y317F mutants stimulated motility substantially; although stimulation was observed by both of these mutants in certain experiments, it was not found to be statistically significant when all experiments were analyzed ($P > 0.1$ by ANOVA). The triple mutant, Shc 3YF, was unable to induce cell motility on either matrix (Fig. 5 a) and appeared to suppress migration somewhat. Therefore, tyrosine phosphorylation of both residues 239/240 and 317 appear to play a critical role in this response.

Shc's phosphotyrosine binding domains, the PTB and the SH2 domains, are known to be crucial for mitogenic signaling through growth factor receptors (Gotoh et al., 1995; Ricketts et al., 1996; Thomas and Bradshaw, 1997). Phosphotyrosines on activated tyrosine kinase receptors can be bound by these domains, thereby localizing Shc to the receptor (Bonfini et al., 1996). To determine whether these domains might also be important for integrins to signal through Shc, cells were transfected with varying amounts Shc cDNAs containing point mutations selectively designed to inhibit function of these domains. Shc S154P contains a loss of function mutation in the PTB domain that prevents interaction with activated insulin receptors (Ricketts, 1999, and Gustafson, T., personal communication). Shc R401L contains a mutation in the conserved phosphotyrosine binding FLVR motif of the Shc SH2 domain, rendering its SH2 domain non-functional (Mayer et al., 1992). Mutation of the PTB domain (ShcS154P)

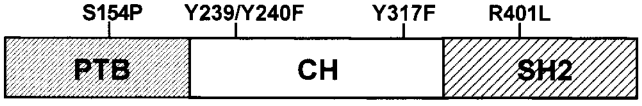

Figure 4. Schematic of Shc constructs. Full-length p52 Shc is depicted. Gray, white, and hatched regions represent the NH_2-terminal PTB domain, the CH domain, and the SH2 domain respectively. The point mutations used are indicated above the schematic. Mutants are identified by S154P, Y239/Y240F, Y317F, Y239/Y240/Y317F (Shc 3YF), and R401L.

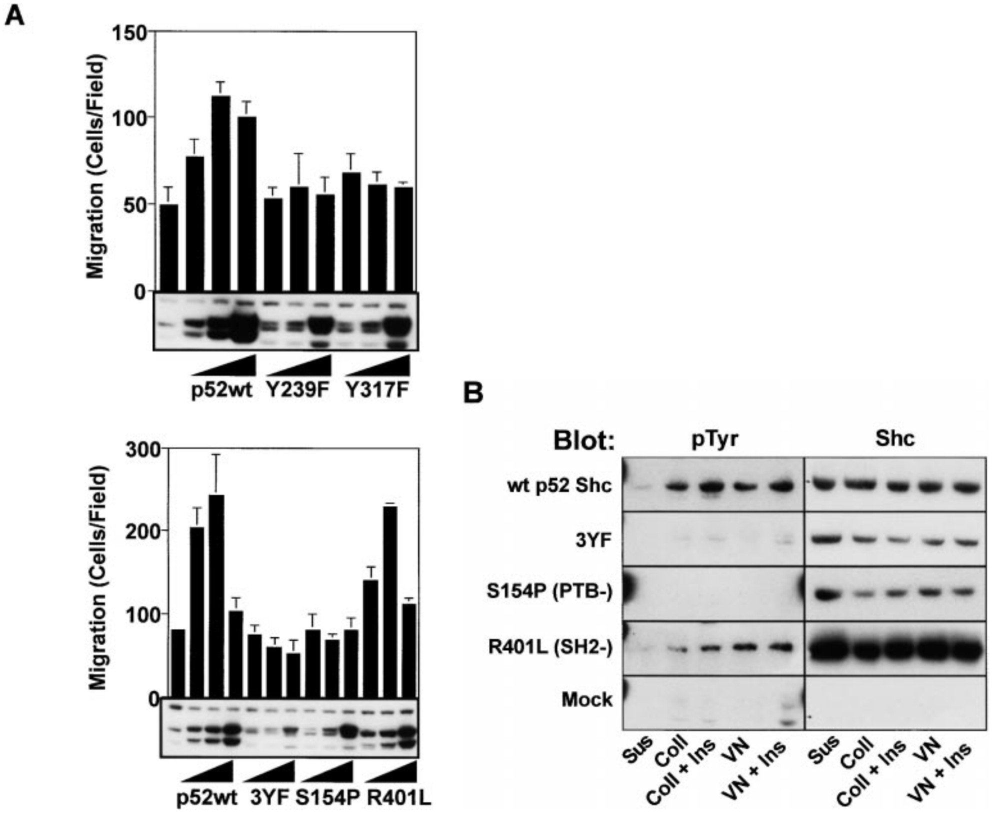

Figure 5. Mutational analysis of Shc-stimulated migration. (A) Cos-7 cells were transfected with increasing amounts of the indicated Shc cDNAs (0.1, 0.3, and 3.0 μg/10-cm plate), serum deprived and assessed for haptotaxis towards collagen. Each bar represents the mean ± SE of a representative experiment. Lysates were generated from the cells used in these experiments and analyzed by immunoblotting with polyclonal anti-Shc antibodies. His-tagged Shc can be discerned from endogenous Shc in the Western blot by its slightly retarded mobility on the gel. The 0.3 μg/plate condition was repeated at least five times for all the mutants with similar results. Cell migration induced by both p52wt and R401L was found to be highly statistically significant ($P < 0.01$ by ANOVA). (B) tyrosine phosphorylation pattern of Shc mutants. Cells were either held in suspension or plated on collagen or vitronectin. As a positive control, cells were stimulated with insulin as indicated. His-tagged Shc was isolated after lysis in RIPA buffer by incubation with nickel agarose beads and analyzed by Western blotting with anti-phosphotyrosine. Blots were then stripped and reprobed with pAb Shc.

blocked Shc's ability to promote haptotaxis (Fig. 5 A). In contrast, the Shc SH2 domain appeared to be dispensable for migration, since ShcR401L consistently promoted migration as efficiently as wild-type Shc (Fig. 5 A). Analysis of six independent experiments confirmed that the migration stimulated by Shc R401L was statistically significant ($P < 0.01$ by ANOVA). The integrin-stimulated tyrosine phosphorylation pattern of these Shc constructs correlated with their ability to promote cell motility. For example, wild-type and Shc R401L were phosphorylated in response to cell adhesion while Shc 3YF and Shc S154P were not. Once again, no differences were observed between the migratory or tyrosine phosphorylation responses on collagen and vitronectin in these studies (Fig. 5 B). In summary, expression of p52 Shc promotes motility of Cos-7 cells, and tyrosine phosphorylation and the PTB domain are required for this response.

Shc Is Required for Haptotaxis of a Metastatic Tumor Cell

To establish whether Shc might be required for cell migration in general, experiments were performed using the constitutively migratory pancreatic carcinoma cell, FG-M. These cells, selected for their migratory properties in vitro, acquired a metastatic phenotype in vivo (Klemke et al., 1998). Shc was constitutively tyrosine phosphorylated in serum-deprived FG-M cells, even when cells are held in suspension (Fig. 6). This is in contrast to Cos-7 cells, which displayed adhesion-dependent Shc phosphorylation. Therefore, we expressed migration incompetent forms of Shc (Shc 3YF and Shc S154P) in FG-M cells to determine if Shc was required for the migration of these cells. As

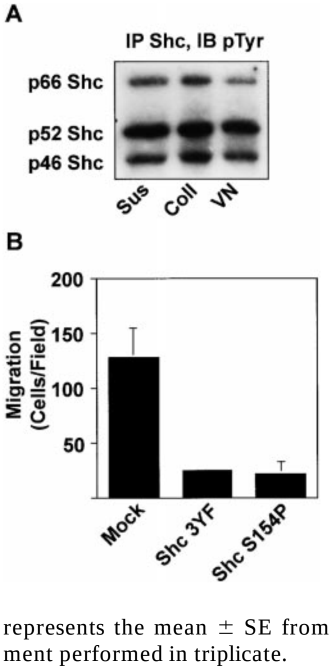

Figure 6. Shc requirement for haptotaxis in a metastatic cell line. (A) Shc is constitutively phosphorylated in human FG-M pancreatic carcinoma cells. Serum-deprived FG-M cells were either held in suspension or plated on extracellular matrix proteins as indicated for 30 min. Cells were then lysed and analyzed by immunoblotting with anti-phosphotyrosine as described in Materials and Methods. (B) FG-M cells require Shc tyrosine phosphorylation and the Shc PTB domain for haptotaxis. FG-M cells were transfected as indicated and assayed for haptotaxis on vitronectin by counting the number of transfected cells/high powered field. Each bar represents the mean ± SE from a single representative experiment performed in triplicate.

shown in Fig. 6, haptotaxis in FG-M cells was highly Shc-dependent, since expression of either Shc 3YF or Shc S154P dramatically inhibited haptotaxis towards vitronectin (by 76 and 83%, respectively). These results suggest that Shc is required for the spontaneous, haptotactic phenotype of these cells.

Shc Is Not Required for Chemotaxis

Based on the findings that Shc appeared to be critical for haptotaxis, studies were performed to investigate the role of Shc in chemotaxis. In these experiments, cells expressing wild-type or mutant forms of Shc were allowed to migrate in collagen coated Boyden chambers towards a gradient of EGF. The EGF response was quantitated by dividing the number of migratory cells observed in the presence vs the absence of EGF for each experimental condition. In mock transfected cells, addition of EGF-stimulated a 2.6-fold increase in migration relative to that observed in its absence. Both wt Shc and Shc R401L substantially increased the number of migratory cells observed in the absence of growth factor, as discussed above. Stimulation of the EGF receptor increased this number further, generating an EGF response of approximately twofold. Surprisingly, Shc was not required for EGF-stimulated motility since expression of either Shc 3YF or Shc S154P failed to block EGF-induced increases in cell migration (Fig. 7). Similar results were observed when IGF-1 was used to stimulate migration (data not shown). Therefore, Shc appears to play a preferential role in haptotaxis as it is not required for the chemotactic response of these cells.

Shc Promotes Growth Factor–induced Mitogenesis Yet Fails to Stimulate Anchorage-dependent DNA Synthesis

The role of Shc in mitogenic signaling by growth factor receptors is well established (Bonfini et al., 1996). In nontransformed cells, integrin ligation and Shc phosphorylation is required for ERK activation and cell cycle progression even in the presence of growth factors (Wary et al., 1996). That Shc was able to mimic growth factor effects on cell migration suggests it may also regulate DNA synthesis under conditions of serum deprivation. To test this possibility, Cos-7 cells were transfected with either wild-type or mutant forms of Shc and then assessed for BrdU incorporation in the absence of exogenous growth factor addition. As shown in Fig. 8, Shc did not regulate DNA synthesis under these growth conditions as wild-type or mutant forms Shc failed to influence BrdU incorporation. Thus, in the absence of exogenous growth factors, Shc expression was not sufficient to promote proliferation even though it was able to induce haptotactic cell migration under these conditions.

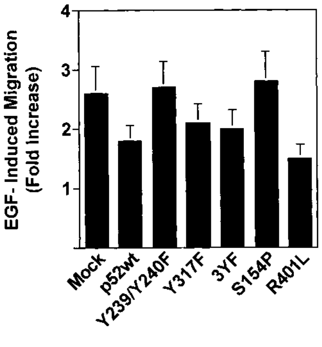

Figure 7. Shc is not required for EGF-stimulated cell migration. Cos-7 cells were transfected with cDNAs encoding Shc mutants as indicated, serum deprived, and assayed for migration in the presence of 100 ng/ml EGF as described in Materials and Methods. The data are depicted as relative migration, with the migration induced by the indicated forms of Shc in the absence of EGF defined as one. Each bar represents the mean ± SE of four independent experiments performed in duplicate. None of the differences between the groups were found to be statistically significant.

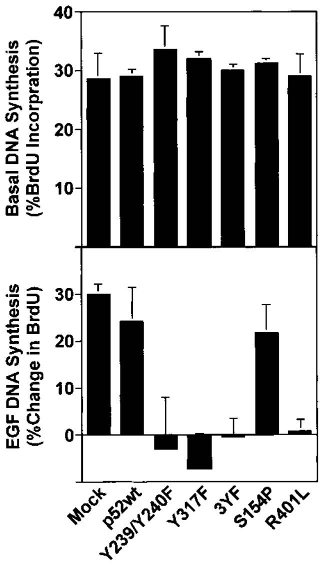

Figure 8. Shc is required for EGF to stimulate anchorage-dependent DNA synthesis. Cos-7 cells were transfected with the indicated cDNAs, serum deprived and assayed for BrdU incorporation in either the absence (top) or the presence of 100 ng/ml EGF (bottom). The data are presented as the percentage of transfected cells incorporating BrdU (top) or as percent increase in BrdU incorporation stimulated by the addition of EGF (bottom). Each bar represents the mean ± SD of three independent experiments performed in triplicate.

Whereas Shc does not influence anchorage-dependent DNA synthesis, previous studies have documented that Shc is crucial for proliferation (Bonfini et al., 1996). Interestingly, the Shc requirements for mitogenesis and haptotaxis are not identical. As shown in Fig. 8, mutation of either tyrosines 239/240 or 317 alone or in combination completely inhibited EGF's ability to increase DNA synthesis, suggesting non-redundant function of these sites for cell cycle progression. Expression of Shc R401L, which induced haptotaxis to the same extent as wild-type Shc, inhibited EGF-stimulated DNA synthesis. In contrast to its effect on haptotaxis, Shc S154P did not affect EGF-stimulated proliferation. Thus, the Shc SH2 domain selectively transduces mitogenic signals in response to EGF, while the PTB domain is critical for haptotaxis.

Discussion

Cell migration and proliferation are required for wound healing, embryonic development, and angiogenesis. The observation that, during these processes, cells can either migrate in the absence of proliferation or proliferate without migrating necessitates the existence of signaling pathways that distinguish between these two responses (Henderson and Copp, 1997; Martin, 1997; Perris, 1997; Risau, 1997). In this report evidence is provided that the adaptor protein, p52 Shc, participates in this decision-making process. Under growth factor–limiting conditions, Shc was required for haptotaxis, but did not influence DNA synthesis. In contrast, when growth factors were present, Shc was crucial for proliferation, but failed to impact cell migration. Shc's phosphotyrosine binding domains were differentially required for these responses; the PTB transduced migratory signals, while the SH2 was required for mitogenesis.

Shc's signaling capacity is regulated by tyrosine phosphorylation. After activation of growth factor receptors and cytosolic tyrosine kinases, Shc becomes phosphorylated (McGlade et al., 1992; Pelicci et al., 1992) on residues 239/240 and 317 within the CH domain and binds Grb2 (Rozakis-Adcock et al., 1992; Salcini et al., 1994; Gotoh et al., 1996; van der Geer et al., 1996), ultimately resulting in the activation of Ras and ERK. However, the biological role of these residues in integrin signaling remains unclear. Recent reports have demonstrated a requirement for Shc Y317 for integrin-mediated ERK activity (Wary et al., 1996), while another report suggested that integrins can stimulate the ERK pathway independently of Y317 (Schlaepfer et al., 1998). In the current report, mutation of either of these sites alone resulted in a loss of Shc's ability to stimulate migration, suggesting that integrins can utilize both of these sites. Tyrosines 239/240 and 317 were crucial for EGF-stimulated mitogenesis in Cos-7 cells, in agreement with findings of others that these sites couple to distinct effectors in mitogenic signaling (Gotoh et al., 1996, 1997). In summary, Shc tyrosine phosphorylation does not distinguish between proliferative and cell migratory signaling pathways.

The observation that cytoskeletally associated kinases phosphorylate Shc (McGlade et al., 1992; Schlaepfer et al., 1998; Wary et al., 1998) suggests that recruitment of Shc to the actin-associated cytoskeleton is an important step in integrin signaling. Recent studies defined a role for the SH3 domain of the Src family member Fyn in this process (Wary et al., 1998). Presumably, the Fyn SH3 domain interacts with proline-rich regions in the CH domain of Shc. The PTB domain may also contribute to Shc recruitment. The amino-terminal domain of Shc was reported to mediate the association of this adaptor protein to an actin-rich cellular fraction (Thomas et al., 1995). In agreement with this report, we find that mutation of the PTB domain abolishes integrin-stimulated Shc tyrosine phosphorylation; the SH2 domain is dispensable for this process. The PTB can bind phospholipids (Zhou et al., 1995) which enhances Shc phosphorylation by cSrc (Sato et al., 1997). Taken together, these observations suggest a model in which the PTB domain localizes Shc to the membrane where it becomes phosphorylated by cytoskeletally associated tyrosine kinases, such as Src, Fyn and/or FAK after integrin ligation, which ultimately results in cell migration.

While Shc-dependent cell migration and proliferation depend upon tyrosine phosphorylation, these processes can be distinguished by their differential requirement for Shc's phosphotyrosine binding domains. The SH2 domain is selectively required for proliferation, as mutation of this domain dramatically inhibited EGF-stimulated DNA synthesis but had no effect on motility, or integrin-induced Shc phosphorylation. The PTB domain, in contrast, mediated tyrosine phosphorylation of Shc in response to integrin ligation and haptotactic cell migration. These findings may be biologically relevant for a number of processes. For example, Shc may play an important role during neural crest cell migration, when cells migrate along a haptotactic gradient without proliferating (Henderson and Copp, 1997) or during wound healing when fibroblasts migrate in a fibrin-rich ECM (Martin, 1997). However, when cells reach areas of high growth factor concentration, Shc may facilitate the cell proliferation response. Activation of the EGF receptor promotes the redistribution of Shc from a perinuclear to a plasma membrane localization (Lotti et al., 1996). EGF-R phosphorylation is required for this effect, presumably because receptor activation creates consensus binding sites for Shc's phosphotyrosine binding domains (Lotti et al., 1996). Thus, when growth factors are present, Shc may be recruited from areas where it transduces haptotactic signals, such as integrin containing focal contacts, to regions of the membrane containing growth factor receptors where it participates in mitogenic signaling.

In summary, proliferation and motility are controlled by adhesion proteins and growth factors associated with the ECM (Martin, 1997). Integrin and growth factor receptor signals signal through the adaptor protein Shc to regulate these responses (Bonfini et al., 1996; Wary et al., 1996, 1998). We find that migratory and proliferative signals also bifurcate at the level of Shc. This divergence is characterized by differential requirements of the Shc PTB domain and the Shc SH2 domain.

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References

Anand-Apte, B., B.R. Zetter, A. Viswanathan, R.G. Qiu, J. Chen, R. Ruggieri, and M. Symons. 1997. Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. *J. Biol. Chem.* 272:30688–30692.

Blaikie, P., D. Immanuel, J. Wu, N. Li, V. Yajnik, and B. Margolis. 1994. A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J. Biol. Chem.* 269:32031–32034.

Bonfini, L., E. Migliaccio, G. Pelicci, L. Lanfrancone, and P.G. Pelicci. 1996. Not all Shc's roads lead to Ras. *Trends Biochem. Sci.* 21:257–261.

Cowley, S., H. Paterson, P. Kemp, and C.J. Marshall. 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell.* 77:841–852.

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

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

Gotoh, N., M. Toyoda, and M. Shibuya. 1997. Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation. *Mol. Cell. Biol.* 17:1824–1831.

Henderson, D.J., and A.J. Copp. 1997. Role of the extracellular matrix in neural crest cell migration. *J. Anat.* 191:507–515.

Huttenlocher, A., R.R. Sandborg, and A.F. Horwitz. 1995. Adhesion in cell migration. *Curr. Opin. Cell Biol.* 7:697–706.

Klemke, R.L., S. Cai, A.L. Giannini, P.J. Gallagher, P. de Lanerolle, and D.A. Cheresh. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137:481–492.

Klemke, R.L., J. Leng, R. Molander, P.C. Brooks, K. Vuori, and D.A. Cheresh. 1998. CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J. Cell Biol.* 140:961–972.

Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin $\alpha v\beta 5$-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell.* 84:359–369.

Lotti, L.V., L. Lanfrancone, E. Migliaccio, C. Zompetta, G. Pelicci, A.E. Salcini, B. Falini, P.G. Pelicci, and M.R. Torrisi. 1996. Sch proteins are localized on endoplasmic reticulum membranes and are redistributed after tyrosine kinase receptor activation. *Mol. Cell. Biol.* 16:1946–1954.

Mainiero, F., A. Pepe, K.K. Wary, L. Spinardi, M. Mohammadi, J. Schlessinger, and F.G. Giancotti. 1995. Signal transduction by the alpha 6 beta 4 integrin: distinct beta 4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:4470–4481.

Martin, P. 1997. Wound healing—aiming for perfect skin regeneration. *Science.* 276:75–81.

Mayer, B.J., P.K. Jackson, R.A. Van Etten, and D. Baltimore. 1992. Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo. *Mol. Cell. Biol.* 12:609–618.

McGlade, J., A. Cheng, G. Pelicci, P.G. Pelicci, and T. Pawson. 1992. Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 89:8869–8873.

Nolan, M.K., L. Jankowska, M. Prisco, S. Xu, M.A. Guvakova, and E. Surmacz. 1997. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. *Intl. J. Cancer.* 72:828–834.

Olson, M.F., A. Ashworth, and A. Hall. 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science.* 269:1270–1272.

Pages, G., P. Lenormand, G. L'Allemain, J.C. Chambard, S. Meloche, and J. Pouyssegur. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA.* 90: 8319–8323.

Pelicci, G., S. Giordano, Z. Zhen, A.E. Salcini, L. Lanfrancone, A. Bardelli, G. Panayotou, M.D. Waterfield, C. Ponzetto, and P.G. Pelicci. 1995. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene.* 10:1631–1638.

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

Perris, R. 1997. The extracellular matrix in neural crest-cell migration. *Trends Neurosci.* 20:23–31.

Ricketts, W.A., J.H. Brown, and J.M. Olefsky. 1999. Pertussis toxin sensitive and insensitive thrombin signaling to shc and mitogenesis are mediated through distinct pathways. *Mol. Endocrinol.* In press.

Ricketts, W.A., D.W. Rose, S. Shoelson, and J.M. Olefsky. 1996. Functional roles of the Shc phosphotyrosine binding and Src homology 2 domains in insulin and epidermal growth factor signaling. *J. Biol. Chem.* 271:26165–26169.

Risau, W. 1997. Mechanisms of angiogenesis. *Nature.* 386:671–674.

Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, and P.G. Pelicci. 1992. Association of the Shc and Grb2/Sem5 SH2–containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature.* 360:689–692.

Salcini, A.E., J. McGlade, G. Pelicci, I. Nicoletti, T. Pawson, and P.G. Pelicci. 1994. Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene.* 9:2827–2836.

Sato, K., N. Gotoh, T. Otsuki, M. Kakumoto, M. Aoto, A.A. Tokmakov, M. Shibuya, and Y. Fukami. 1997. Tyrosine residues 239 and 240 of Shc are phosphatidylinositol 4,5-bisphosphate-dependent phosphorylation sites by c-Src. *Biochem. Biophys. Res. Commun.* 240:399–404.

Schlaepfer, D.D., K.C. Jones, and T. Hunter. 1998. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol. Cell. Biol.* 18:2571–2585.

Thomas, D., and R.A. Bradshaw. 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.Sos1 binding site. *J. Biol. Chem.* 272:22293–22299.

Thomas, D., S.D. Patterson, and R.A. Bradshaw. 1995. Src homologous and collagen (Shc) protein binds to F-actin and translocates to the cytoskeleton upon nerve growth factor stimulation in PC12 cells. *J. Biol. Chem.* 270: 28924–28931.

van der Geer, P., S. Wiley, G.D. Gish, and T. Pawson. 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.

Wary, K.K., F. Mainiero, S.J. Isakoff, E.E. Marcantonio, and F.G. Giancotti. 1996. The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell.* 87:733–743.

Wary, K.K., A. Mariotti, C. Zurzolo, and F.G. Giancotti. 1998. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell.* 94:625–634.

Zhou, M.M., K.S. Ravichandran, E.F. Olejniczak, A.M. Petros, R.P. Meadows, M. Sattler, J.E. Harlan, W.S. Wade, S.J. Burakoff, and S.W. Fesik. 1995. Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature.* 378:584–592.